Advances in Life Science and Technology
ISSN 2224-7181 (Paper) ISSN 2225-062X (Online)
Vol.27, 2014

Real-Time PCR and Its Application in Plant Disease Diagnostics

Kumlachew Alemu
Department of Plant Science, Assosa University, P.O.Box 18, Assosa, Ethiopia
E-mail of corresponding Author: kum.alemu@gmail.com

Abstract
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. Generally, real-time PCR technologies open increasing opportunities and a significant role in better understanding of the dynamics of plant pathogenic microorganism and, thereby allow better management of the diseases.
Key words: Diagnostics, high-throughput, Pathogen, Quantification, Real-time PCR

1. Introduction

Diseases in plants cause major production and economic losses in agricultural industry 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 required to apply treatments, undertake agronomic measures or proceed with eradication practices. 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 (Lopeze *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).
Molecular diagnostic began to develop a real momentum after the introduction of polymerase chain reaction (PCR) in the mid 1980s and the first PCR based detection of a pathogen in diseased plants was published in the beginning of 1990s. There has been an exponential increase in the number of protocols based on nucleic-acid tools. Nucleic acid amplification and detection are among the most valuable techniques used in biological research today (Paplomatas, 2006). To date an increasing number of diagnostic laboratories is adapting molecular methods for routine detection of pathogens. 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 and 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. However, while the specificity and sensitivity of detection of pathogens are greatly improved and pathogen detection is becoming simpler and faster, there are still major challenges, technical and economic nature, which need to be addressed to ensure the emergence of reliable detection system for routine applications. This paper gives an overview about recent advancement in molecular diagnosis of plant

disease focusing on the application of real-time PCR in detection and diagnosis of plant pathogenic microorganisms.

2. The Real-Time PCR

2.1. Principle of Real-Time PCR

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction 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 to run the PCR product out on a gel after the reaction (Paplomatas, 2006; Capote *et al*., 2012).

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 labeled 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).

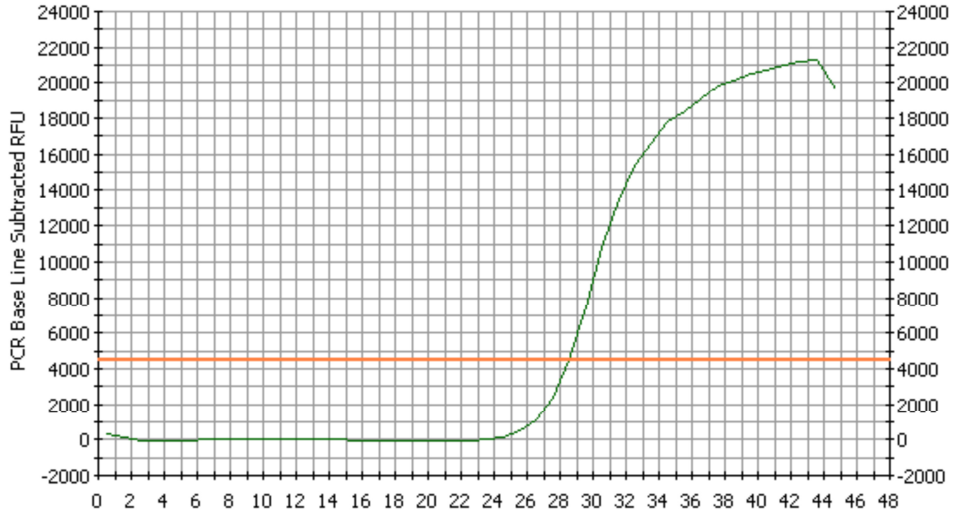

Figure 1: Amplification plot

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 technologies

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 group into amplicon sequence non-specific method which based on the use of doubled-stranded DNA binding dyes, such as SYBR Green, and sequence specific methods which based on fluorescent labeled probes such as TaqMan, Molecular Beacons or Scorpions (Schena *et al*., 2004; Capote *et al*., 2012).

2.2.1. Amplicon sequence non-specific methods

Included in this group are detection methods based on the use of dyes that emit fluorescent light when intercalated into double-stranded DNA (dsDNA). The most utilized fluorescence intercalating dye is SYBR Green I as it has high affinity for double-stranded DNA although other intercalating dyes, such as ethidium bromide have been used (Capote *et al*., 2012). In solution, the unbound dye exhibits very little fluorescence, but this is enhanced upon DNA-binding (Figure 2). As a consequence, the fluorescence is proportional to the amount of total dsDNA in the reaction. Since these dyes do not discriminate between the different dsDNA molecules, in a PCR reaction, the formation of non-specific amplicons, as well as of dimers, must be prevented by accurate primer design and condition optimisation. At the end of the reaction, products of different length and/or sequence can be observed as distinct fluorescent peaks by heating the reaction from 30–40 ^{o}C to 95 ^{o}C whilst continuously monitoring the fluorescence (melting curve analysis). In fact, plotting the first negative derivative of fluorescence vs. temperature, the point at which dsDNA melts will be observed as a drop (peak) in fluorescence. If a single peak representing the specific products is observed, SYBR Green I is a simple and reliable low-cost method for monitoring PCR amplicons and for quantifying template DNA. However, since different amplicons will melt at different temperatures, melting curve analyses also enable the use of SYBR Green I in multiplex detection (Schena *et al*., 2004).

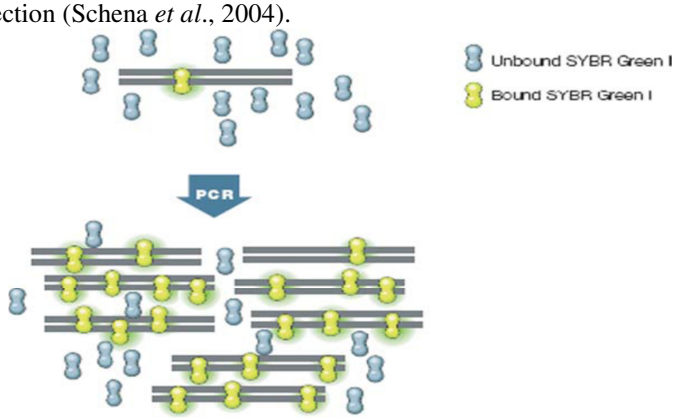

Figure 2: DNA binding dyes in Real time PCR. Fluorescence dramatically increases when the dye molecule binds to DNA

2.2.2. Amplicon sequence specific methods

There are several amplicon sequence specific detection methods based on the use of oligonucleotide probes labelled with a donor fluorophore and an acceptor dye (quencher). This includes TaqMan, Molecular beacons, and Scorpion PCR. The probes used to monitor DNA amplification in a PCR are cleaved in the reaction (TaqMan) or undergo a conformation change in the presence of a complementary DNA target (Molecular beacons and Scorpion- PCR) that separate fluorophore and quencher. In TaqMan, the fluorophore is quenched by FRET, whereas in Molecular beacons and Scorpion-PCR, fluorescence quenching is proximal, due to the close contact of fluorophore and quencher (Schena *et al*., 2004).

2.2.2.1. The TaqMan probe method

Fluorescent probes are pieces of DNA complimentary to your gene of interest that are labeled with a fluorescent dye. The simplest and most commonly used type of probe is the TaqMan-type probe. The TaqMan probe method utilizes a fluorescently labelled probe that hybridizes to an additional conserved region that lies within the target amplicon sequence. These probes are labeled with a fluorescent reporter molecule at one end and a quencher molecule (capable of quenching the fluorescence of the reporter) at the other. Hence under normal circumstances the fluorescent emission from the probe is low. However during the PCR the probe binds to the gene of interest and becomes cleaved by the polymerase. Hence the reporter and quencher are physically separated and the fluorescence increases (Schaad *et al*., 2003; Capote *et al*., 2012). Fluorescence can be measured throughout the PCR, providing "real-time" analysis of the reaction kinetics and allowing quantification of specific DNA targets. The measurement of fluorescence throughout the reaction eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, facilitating automation of the

technique and large-scale sample processing. Interpretation of the fluorometric data can be presented during the PCR assay (Figure 3), and also facilitates quantification of the amount of sample DNA present in the reaction by ascertaining when (i.e. during which PCR cycle) fluorescence in a given reaction tube exceeds that of a threshold value (Threshold Cycle (Ct)). Comparison between reaction tubes and/or known standards allows quantification of the amount of DNA template present in a given tube (Weller *et al*., 2006).

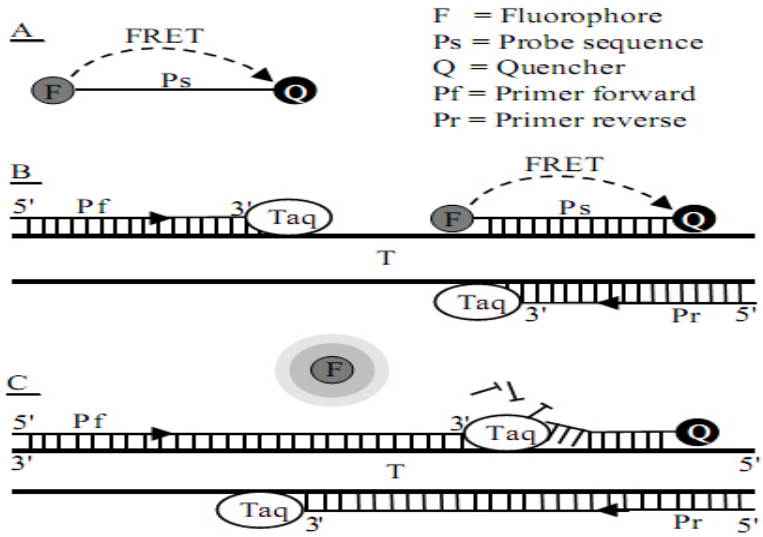

Figure 3: Real-time PCR, TaqMan™ chemistry

This system consists of a single stranded sequence (probe sequence) with a fluorophore and a quencher attached respectively to the 5¢ and the 3¢ end (A). The probe sequence binds to the amplicon during each annealing step of the PCR (B) and generates a signal through the 5¢–3¢ exonuclease activity of the Taq DNA polymerase that degrades the probe sequence and releases both fluorophore and quencher into the solution (C). Once cleaved from the rest of the probe sequence, the 5¢ dye is freed from the quenching effect

2.2.2.2. Molecular beacons

Molecular beacons are fluorescent oligonucleotide probes that are designed to include stem-loop folding. They are complementarity nucleotide sequences that are complementary to the target amplicon. A fluorescent chromophore is attached at the 5′ end of the probe and a quencher molecule is attached at the 3′ end (Figure 4). A stem structure is formed by annealing of the complementary arm sequences that are added on both sides of the probe sequence. When a stem structure is formed, the fluorophore transfers energy to the quencher, and no fluorescence is emitted. However, when the probe hybridizes to the target amplicon during PCR amplification, the fluorophore and quencher become separated from each other and fluorescence can be detected (Schaad and Frederick, 2002).

2.2.2.3. Scorpion-PCR

This assay employ two primers, one of which serves as a probe and contains a stem-loop structure with a 5' fluorescent reporter and 3' quencher, as illustrated in Figure 5. The loop sequence of the Scorpions probe is complementary to an internal portion of the target sequence on the same strand. During the first amplification cycle, the Scorpions primer is extended and the sequence complementary to the loop sequence is generated on the same strand. The Scorpions probe contains a PCR blocker just 3' of the quencher to prevent read-through during the extension of the opposite strand. After subsequent denaturation and annealing, the loop of the Scorpions probe hybridizes to the target sequence by an intra-molecular interaction, and the reporter is separated from the quencher. The resulting fluorescent signal is proportional to the amount of amplified product in the sample. Comparison among Scorpion primers and alternative chemistries showed that TaqMan probes have high backgrounds and moderate signal strength, molecular beacons have low background fluorescence and low signal strength, Scorpion primers have low backgrounds and high signal strength (Thelwell *et al*., 2000).

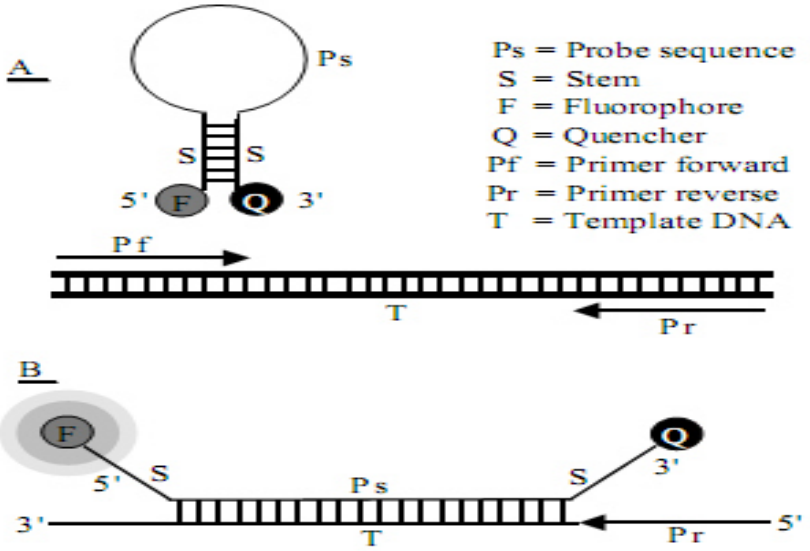

Figure 4: Molecular beacon

This system consists of a hairpin loop structure (probe sequence), a 6-base-long stem that hold the probe sequence in the hairpin configuration and a fluorophore and a quencher linked, respectively, to the 5¢ and the 3¢ end of the stem (A). Molecular beacons generate signals when hybridise to the amplified template DNA and distance of the 5¢ dye molecule from the quenching molecule is great enough to have a specific increase of fluorescence (B). This hybridisation is favoured over the intramolecular binding of the complementary target strand, however, if there is no template DNA the hairpin configuration is restored and the fluorescence quenched (Schena *et al*., 2004)

A further improvement of the 'stem-loop' format has been achieved with the 'duplex format'. In this format the probe element has a fluorophore attached at its 5¢-end and is annealed to a complementary oligonucleotide bearing a quencher at the 3¢-end (Figure 6). The mechanism of action is quite similar to the stem-loop format, since the intra-molecular probe-target interaction, which is the most important feature of the Scorpion system, is maintained in both formats. However, in standard Scorpions the quencher and fluorophore remain within the same strand of DNA and some quenching can occur even in the open form. In duplex format, the quencher is on a different oligonucleotide and separation between the quencher and fluorophore is greatly increased, thus decreasing the quenching when the probe is bound to the target (Solinas *et al*., 2001).

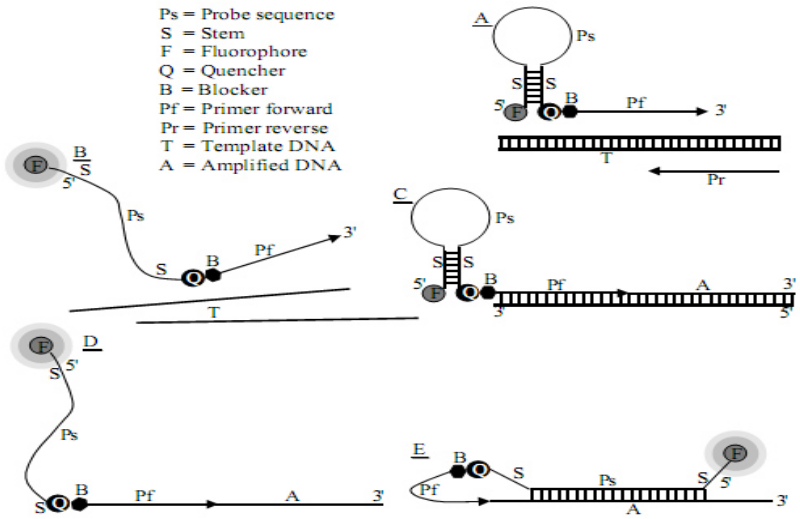

Figure 5: Scorpion PCR (stem loop format).

This system is similar to Molecular beacons, however the hairpin loop is linked to the 5¢ end of a primer via a PCR blocker (A). Step B: Initial denaturation of target and scorpion sequence. Step C: Annealing of the primer element of the Scorpion to the target DNA and extension of a new DNA fragment to which Scorpion remains

attached. Step D: Denaturation of DNA. Step E: During annealing extension the Scorpion probe binds to its complement within the same strand of DNA. This hybridisation event opens the hairpin loop so that fluorescence is no longer quenched and a specific increase of fluorescence is achieved (Schena *et al*., 2004)

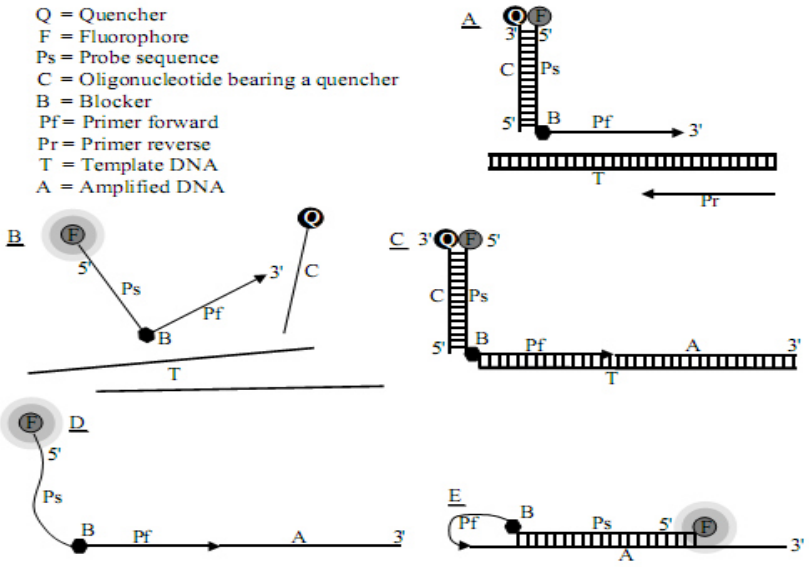

Figure 6: Scorpion PCR (duplex format).
The unimolecular probing mechanism is basically the same as in the stem loop-format (Figure 5), however the quencher is on a different oligonucleotide chain (A). Step B: Initial denaturation. Step C: Annealing of the primer element of the Scorpion to the target DNA and extension of a new DNA fragment to which Scorpion remains attached. Step D: Denaturation of DNA. Step E: During annealing extension the Scorpion probe binds to its complement within the same strand of DNA. This hybridisation event prevents annealing of the probe sequence with the complementary oligonucleotide bearing the quencher so that fluorescence is no longer quenched and a specific increase of fluorescence is achieved (Schena *et al*., 2004).

2.3. 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 (Lopeze *et al*., 2003). Too low sensitivity often leads to false negatives. Thus, a high degree of diagnostic accuracy is characterized by the ability to detect, true and precisely the target micro organism from a sample without interference from non target components. The high degree of sensitivity of molecular methods made pre-symptomatic detection and quantification of pathogens possible (Degefu, undated). 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). The detection is based on the measurement of the fluorescence emitted by probes incorporated into the newly formed PCR product, or alternatively released into the buffer during the amplification of the PCR product. Intercalating agents and fluorogenic probes are the two main types of molecules currently used to detect PCR amplification in real-time.

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 (Lopeze *et al*., 2003). In a study carried out on four pea thioredoxin h (TRXh) encoding genes, Montrichard *et al*. (2003) noticed that real-time PCR yielded weaker signals than expected from northern blot analyses. This observation was explained by a cross-hybridization of the probe to the RNA encoding another isoform during the northern blot procedure. Indeed, in contrast to techniques requiring the hybridization of nucleic acids several hundred base pairs long, such as cDNA-based microarray and northern blotting, short oligonucleotide-mediated real-time PCR guarantees a high specificity in the detection of the target sequence. In fact, specificity is achieved by the use of two target sequence-specific oligonucleotides, and this can be enhanced by increasing the number of oligonucleotides nested within the initial amplification product. In this respect, FRET-mediated probes seem to ensure a higher specificity than SYBR green® (Shimada *et al*., 2003). In any case, specificity of the process can be checked after completion of the PCR run, by testing the nature of the amplified product with gel electrophoresis, melting curves, and sequencing data.

Quantification: Nucleic acid quantification meant the addition of radio-labeled deoxythymidine triphosphate (dTTP) to cell cultures or one of many possible in vitro experimental preparations and measuring their incorporation into nucleic acids by TCA (trichoroacetic acid) precipitation. Although radioactive incorporation is a quantitative technique and gave the investigator an idea of the global changes in the nucleic acid population of their experimental system, it was not satisfactory for identifying or quantifying specific genes or transcripts (Dorak, 2006). 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. Application of Real Time PCR in Plant Disease Diagnostics

3.1. 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 health care, agriculture and environmental monitoring including the fight against bioterrorism in plant disease management (Schaad and Frederick, 2002, Schaad *et al*., 2003).Today, diagnosticians have an array of methodologies to help in this respect. 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 (López *et al*., 2003).

In recent years, molecular techniques of plant disease detection have been well established. With this regard the development of real time-PCR techniques plays indispensable role. From the middle of the 1990s a wide range of PCR assays were developed and applied to the diagnosis of infectious diseases, as well to the improved detection of pathogens. 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 (Lopeze *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 such as SYBR R Green; showed high sensitivity and specificity for the detection plant pathogenic microorganisms.

3.2. 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 (Degefu, undated). Quantification of inoculum is essential mostly for soil-borne

pathogens although it is well established that quantity of initial inoculum has a critical importance in the subsequent epidemic caused by pathogens disseminated by the wind (Paplomatas, 2006).

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 micro organisms 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 (Degefu, undated; Schena *et al*., 2004).

The process of quantifying target DNA has recently been simplified considerably with the advent of real-time PCR. This method avoids the usual need for post-reaction processing, as the amplified products are detected by a built-in fluorimeter as they accumulate. This is done by using non-specific DNA binding dyes (e.g. SYBR Green) or fluorescent probes that are specific to the target DNA (Wittwer *et al*., 1997). The principle underlying real-time PCR is that the larger the amount of target DNA present in the sample being tested, the quicker the reaction progresses and enters the exponential phase of amplification. 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 analyze 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).

Real-time PCR assays aiming at quantifying the level of plant infection by a pathogen have been increasing for the last few years. Most of them rely on the relative quantification of two specific plant and pathogen DNA sequences. They are faster, more specific and more sensitive when compared with traditional protocols based on symptom recording or on conidiophore or colony counting (Winton *et al*., 2003), and, most importantly, may be transposed to virtually every pathosystem. Classical detection methods require a labor-intensive culture and pathogenicity test on tomato seedlings. However, real-time PCR has been shown to enable the quantitative detection of *R. solanacearum* in a rapid and reliable manner, thus providing an improved alternative assay that could be implemented on a large scale (Weller *et al*., 2000).

Likewise, food contamination by mycotoxins is of great concern, since many have been found to be carcinogenic and they are not easily removed during food processing. However, since toxin abundance does not correlate with fungal contamination, but is linked to the toxinogenic properties of each strain, real-time PCR detection assays targeted at genes involved in toxinogenesis have been developed for trichotecene-producing Fusarium and aflatoxin-producing Aspergillus species (Schnerr *et al*., 2001). Petit *et al*., (2003) recently implemented a refinement of this technique based on the quantification of the nor1 mRNA, which directly addresses aflatoxin biosynthesis in infected wheat. As many countries are becoming more and more concerned about food safety, the market for such applications is growing rapidly. Real-time PCR application in fundamental studies is still lagging behind, and only a few real-time PCR-based pathogenicity assays have been reported in this field (Hiriart *et al*., 2003). Most of the currently used resistance tests rely on visual assessment of the symptoms and spore or colony counting.

3.3. Determination of Viability of Pathogen

Nucleic acid based detection methods currently applied in pathogen detection are based on nucleic acid hybridization or PCR. These methods can be designed to detect either DNA or mRNA. Whereas DNA based detection method is often more straightforward than that of mRNA, the stability of DNA leads to the possibility that DNA based methods yield positive results from non-viable or dead pathogens. 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 consider enrichment culturing instead of direct PCR. While the system allows the detection of only viable cells and helps

in elimination of possible PCR inhibitors, it is not appropriate approach for quantitative assay. Therefore, the lack of ability to distinguish between viable and dead cells, and the lack of sample preparation methods that do not involve enrichment culturing are currently limiting the implementation of real-time PCR for routine diagnostic use. Molecular methods for inferring pathogen viability focus on detecting mRNA in a sample as mRNA species are believed to be labile with a very short half life after cell death. However, although it is in theory a more accurate indicator of viable micro organisms there has been report of poor correlation between the two variables (Sung *et al*., 2004).

3.4. Detection of 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. The methodological limitations however, are in many cases the reasons for developing simplex or assays only including few targets. 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. Development of real-time multiplex PCR for diagnostic applications is a recent history. In plant pathology the method was applied for identifying nematode bacterial and fungal DNA from pure cultures. Schena *et al*. (2004) employed a real-time multiplex PCR approach based on TaqMan PCR to simultaneously identify and detect these four Phytophthora species. Specific primers and probes labelled with FAM (*P. ramorum*), Yakima Yellow (*P. kernoviae*), Rox (*P. citricola*) and Cy5 (*P. quercina*) were designed in different regions of the ras-related protein (Ypt1) gene. A new set of Black Hole Quenchers (BHQ), which dissipate energy as heat rather than fluorescence, were utilized. The method proved to be highly specific in tests with target DNA from 72 Phytophthora isolates (35 species). And they concluded that method, combined with a rapid procedure for DNA extraction, and proved to be rapid, reliable, sensitive and cost effective as multiple pathogens were detected within the same plant extract by using different primer/probe combinations.

3.5. Monitoring of Fungicide Resistance

Fungicides are commonly used to manage plant disease. However the frequent use of fungicides with a single mode of action incurs a high risk of selecting resistance genotype of plant pathogens. To determine level of resistance to fungicides, the most common traditional technique is direct-planting single spore isolates in media amended with various concentrations of fungicide and determining inhibition of growth or/and spore germination. The entire test can take 1 to 3 weeks longer if the time required to isolate the pathogen from infected plant tissue is included. This conventional technique is time consuming but critical since grower relay on the result to decide on fungicide programs for their orchards (Michailides *et al*., 2005).

With the advancement of molecular techniques particularly the real-time PCR techniques resistance development by pathogen to a particular fungicide can be detected easily within a short period of time. Michailides *et al*., (2005) indicated that resistance of Alternaria to azoxystrobin and Benzimidazoles resistance in *M. fructincola* in stone fruit and *M. laxa* isolates in almonds can be easily detected with allele specific real-time PCR. Similarly, Guo *et al*. (2007) employed a real-time PCR assay to evaluate the effects of fungicide treatments (Fandango (prothioconazole 100 g + fluoxastrobin 100 g a.i./l), Input (prothioconazole 160 g spiroxamine 300 g a.i./l) and Prosaro (prothioconazole 125 g + tebuconazole 125 g a.i./l)) on *Mycosphaerella graminicola* leaf blotch in the field, they and indicated that the real-time PCR assay can serve as an alternative method for accurate assessment of the fungicide effects on *M. graminicola* leaf blotch.

4. Conclusion

Detection and accurate identification of plant pathogens is one of the most important strategies for controlling plant diseases to initiate preventive or curative measures. In recent years, molecular techniques of plant disease detection have been well established, particularly the development of real-time PCR technology have resulting in more convenient, effective, and specific assays have opened the door to greater use of these tests for detection

and identification of phytopathogenic microorganisms. Such assays will help growers, crop agronomists, and plant-health professionals not to rely exclusively on symptomatology and permit early detection of pathogenic microorganisms. This new techniques are effective management tools to be used in parallel with knowledge of the crop, understanding the biology of the pathogen and the ecology of the disease. Thus, this tool can be excellent tool to determine the point in time at which control measures should be implemented. The development new technologies and improved methods with reduced cost and improved speed, throughput, multiplexing, accuracy and sensitivity have emerged as an essential strategy for the control of plant diseases. 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.

References

Artz, R.R.E., Avery, L.M., Jones, D.L. and Killham, K. (2006). Potential pitfalls in the quantitative molecular detection of Eschrichia coli 0157:H7 in environmental matrices. *Canadian Journals of Microbiology* 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 physiology* 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. *Molecular and Cellular Probes* 17: 69-75.

Bustin, S.A., (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assay. Journal of Molecular Endocrinology 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. In Cumagun, C.J., (Ed.), 2012. Plant Pathology, (ISBN: 978-953-51-0489-6.

Chilvers, M.I. (2012). Molecular Diagnostics in Plant Disease Diagnostic Clinics… What's the Status? Fungal Genome Biol 2:e102. doi:10.4172/2165-8056.1000e102.

Degefu, Y., undated. Molecular diagnostic technologies and end user applications: potentials and challenges. www.annualreviews.org/doi/abs/10.../annurev.py.26.090188.002205.

Dorak, M. T. (2006). Real-time PCR. Taylor & Francis Group, UK. 362p.

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

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

Guo, .R., Schnieder F. and Verreet, J.A. (2007). A Real-time PCR Assay for Quantitative and Accurate Assessment of Fungicide Effects on Mycosphaerella graminicola Leaf Blotch. *Journal of Phytopathology* 155: 482–487.

Hampton, R.O., Ball, E.M., and De Boer, S.H. (1990). Serological tests for detection of viral and bacterial pathogens. American Phytopathological Society, St. Paul, Minn.

Henson, J.M., and French, R. (1993). The polymerase chain reaction and plant disease diagnosis. Annual *Review of Phytopathology* 31: 81–109.

Hiriart, J.B., Aro, E.M. and Letho, K. (2003). Dynamics of the VIGS-mediated chimeric silencing of the Nicotiana benthamiana ChlH gene and of the tobacco mosaic virus vector. *Molecular Plant Microbe Interactions* 16:99-106.

Keer, J.T. and Bircéh, L. (2003). Molecular methods for assessment of bacterial viability. *Journal of Microbiolal Methods* 53:175-183.

Li, W., Hartung, J.S. and Levy, L. (2006).Quantitative real-time PCR for detection and identificationof species associated with citrus huanglongbing. *Journal of Microbiological Methods* 66:104–115.

Lo´Pez, 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 Journal of Plant Protection* 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. *California agriculture* 59(2): 115-123.

Montrichard, F., Renard, M., Alkhalfioui, F., Duval, F.D. and Macherel, D. (2003). Identification and differential expression of two thioredoxin h isoforms in germinating seeds from pea. *Plant Physiology*132: 1707–1715.

Mumford, R., Boonham, N., Tomlinson, J. and Barker, I. (2006). Advances in molecular phytodiagnostics –new solutions for old problems. *European Journal of Plant Pathology* 116: 1–19.

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

Petit, L., Baraige, F., Balois, A.M., Bertheau, Y. and Fach, P. (2003). Screening of genetically modified organisms and specific detection of Bt 176 maize in flours and starches by PCR-enzyme linked immunosorbant assay. *European Food Research and Technology* 217: 83-89.

Sankaran, S., Mishra, A., Ehsani, R. and Davis, C. (2010). A review of advanced techniques for detecting plant diseases. *Computers and Electronics in Agriculture* 72: 1–13.

Schaad, N.W. and Frederick, R.D. (2002). Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology* 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. *Annual Review of Phytopathology* 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. *European Journal of Plant Pathology* 110: 893–908.

Schnerr, H. Niessen, L. and Vogel, R.F. (2001). Real time detection of the tri 5 gene in Fusarium species by light cycler TM using SYBER Green 1 for continuous monitoring. *International journal of food microbiology* 71: 53-61.

Shimada Y, Goda H, Nakamura A, Takatsuto S, Fujioka S, and Yoshida, S. (2003). Organ-specific expression of brassinosteroid-biosynthetic genes and distribution of endogenous brassinosteroids in Arabidopsis. *Plant Physiology*131:287–297.

Solinas, A., Brown, L.J., McKeen, C., Mellor, J.M., Nicol, J.T.G., Thelwell, N. and Brown, T. (2001). Duplex Scorpion primers in SNP analysis and FRET applications. *Nucleic Acids Research* 29: 96–104.

Sung, K.D., Stern, N.J. and Hiett, K.L. (2004). Relationship of messenger RNA Reverse Transcriptase-Polymerase Chain Reaction signal to Campylobacter spp. viability. *Avian Disease* 48:254-262.

Thelwell, N., Millington, S., Solinas, A., Booth, J. and Brown, T. (2000). Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Research* 28: 3752–3761.

Ward, E., Foster, S. J., Fraaije, B. A. and Mccartney, H. A. (2004). Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology* 145:1-16.

Weller, S.A., Elphinstone, J.G. Parkinson, N. and Thwaites, R. (2006). Molecular Diagnosis of Plant Pathogenic Bacteria. *Arab Journal of Plant Protection* 24: 143-146.

Weller, S.A, Elphinstone, J.G, Smith, N.C., Boonham, N. and Stead, D.E. (2000). Detection of Ralistonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and environmental microbiology* 66: 2853-2858.

Winton, L.M., Manter, D.K., Stone, J.K. and Hansen, E.M. (2003). Comparsion of biochemical, molecular and visual method to quantify Phaeocryptopus gaeumannii in douglas-fir foliage. *Phytopathology* 93: 121-126.

Wittwer, C.T., Herrmann, M.G., Moss, A.A. and Rasmussen, R.P. (1997). Continuous fluorescence monitoring of rapid cycle DNA amplification. *Bio techniques* 22:130-138.

The IISTE is a pioneer in the Open-Access hosting service and academic event management. The aim of the firm is Accelerating Global Knowledge Sharing.

More information about the firm can be found on the homepage:
http://www.iiste.org

CALL FOR JOURNAL PAPERS

There are more than 30 peer-reviewed academic journals hosted under the hosting platform.

Prospective authors of journals can find the submission instruction on the following page: http://www.iiste.org/journals/ All the journals articles are available online to the readers all over the world without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. Paper version of the journals is also available upon request of readers and authors.

MORE RESOURCES

Book publication information: http://www.iiste.org/book/

Academic conference: http://www.iiste.org/conference/upcoming-conferences-call-for-paper/

IISTE Knowledge Sharing Partners

EBSCO, Index Copernicus, Ulrich's Periodicals Directory, JournalTOCS, PKP Open Archives Harvester, Bielefeld Academic Search Engine, Elektronische Zeitschriftenbibliothek EZB, Open J-Gate, OCLC WorldCat, Universe Digtial Library , NewJour, Google Scholar