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Real-Time PCR Applied to Study on Plant Pathogens: Potential Applications in Diagnosis – a Review

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Abstract

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Quantitative real-time PCR (qPCR) technique incorporates traditional polymerase chain reaction (PCR) efficiency with the production of a specific fluorescent signal, measuring the kinetics of the reaction in the early PCR phases and providing quantification of specific targets in various environmental samples. There are an increasing number of chemistries to detect PCR products, which are widely used in plant pathology as they cluster into the amplicon sequence non-specific and sequence-specific techniques. In this review, we illustrate a general description of major chemistries and discuss some considerations for assay development as it applies for a wide range of applications in epidemiological studies. The technique has become the gold standard for early detection of pathogens and a fundamental tool in the research laboratory.

Keywords: bacteria; fungi; oomycetes; phytoplasmas; viroids; viruses; plants; quantification; polymerase chain reaction; qPCR chemistries

One of the most important strategies for controlling plant diseases is accurate and early detection and identification of plant pathogens (Garrido *et al*. 2012); actually, this is the basis of plant disease management. Of particular importance is early detection of pathogens in seeds and plant materials to avoid further spreading and introduction of new pathogens into a growing area where it is not yet present (Acero *et al.* 2011). The accessibility of a fast, sensitive, and accurate method for detection of pathogens to improve disease control decision making is therefore increasingly required. The traditional detection methods based on morphological characteristics often require extensive knowledge of classical taxonomy and are frequently laborious and time-consuming (Capote *et al.* 2012). Moreover, the difficulty of culturing some species *in vitro* and the inability for accurate quantification of the pathogen are other limitations (Goud & Termorshuizen 2003). These limitations have led to the development of molecular approaches with improved accuracy and reliability. Molecular techniques are able to identify non-culturable microorganisms and so provide precise, reliable, and reproducible results, facilitating early disease management decisions (Martin *et al*. 2004).

Polymerase chain reaction (PCR)-based technology is a rapid and sensitive method that offers advantages over the traditional diagnosis methods. First of all, micro-organisms do not need to be cultured; second, it possesses the potential to detect a single target molecule in a complex mixture; and third, these techniques can considerably reduce the time

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needed for diagnosis compared with conventional culturing methods. However, they still require further work to identify the PCR products when southern blot or sequencing are needed (Okubara *et al.* 2005). Conventional PCR (cPCR) has emerged as a main tool for the diagnosis of plant pathogens and has contributed to reducing some problems related to the plant pathogens detection (Martin *et al.* 2000). On the other hand, because of different testing parameters in cPCR assays, optimisation of conditions is very challenging and time consuming (Espy *et al.* 2006). As a result, cPCR techniques have never been extensively used for quantitative analysis of plant pathogens, since they are inaccurate and laborious (Schena *et al.* 2013). However, several cPCR methods have been used for quantitative analysis of plant pathogenic fungi (Hadidi *et al.* 1995; Mahuku & Platt 2002).

The necessity of fast, sensitive, and specific methods to detect pathogens is important to improve decision making in disease control (Lievens *et al.* 2005). Quantitative real-time PCR (qPCR) technology allows accurate detection and/or quantification of pathogens that cannot be extracted or cultured easily from host tissue, or are presented at low inoculum load in samples. In fact, quantification based on culturing techniques is considered relatively inaccurate, while quantification using real-time PCR provides a reliable estimation of the pathogen load (Garrido *et al.* 2009). Also, real-time PCR technology provides conclusive results as it can discriminate between closely related organisms and is therefore a versatile method for the accurate, reliable, and high throughput quantification of target DNA in various biological fields such as botany and genetics (Cooke *et al.* 2007; Schena *et al.* 2013). Nowadays, a wide range of plant pathogens can be detected and quantified by real-time PCR methods in numerous hosts or environmental samples. The present compilation illustrates a general description of four basic real-time PCR chemistries used in plant pathology and examples of applicability of this important technique for routine detection and/or quantification of plant pathogens including viruses, viroids, bacteria, phytoplasmas, fungi, and oomycetes. Some considerations for assay development are discussed.

Real-time PCR techniques

As the name suggests, real-time PCR is a technique used to screen the development of a PCR reaction in real time. It is based on the detection of the fluorescence produced by a reporter molecule which increases as the reaction proceeds. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA or sequence specific probes (Garrido *et al.* 2012). The benefit of real-time PCR compared with cPCR is determined by two main features. Firstly, data are available in real time, on screen, do not require time consuming post-PCR processing (e.g. electrophoresis, colorimetric reaction or hybridisation). Secondly, real-time PCR commonly amplifies the short DNA fragments (70–100 bp), which favours a higher level of efficiency and sensitivity (Garrido *et al.* 2009). Generally, the main advantage of real-time PCR over cPCR is the increased sensitivity and the ability to perform quantitative measurements, making it suitable for studying pathogen biology, epidemiology, and ecology. The advantages of the fluorescence based real-time PCR have revolutionised the approach to PCR-based quantification of nucleic acids (Wittwer *et al.* 2001; Okubara *et al.* 2005). Real-time PCR can successfully quantify the initial specific target by the measurement of the amplification products. This occurs due to the accumulation of the PCR product with each cycle of amplification, and this is the reason why this method is called real-time PCR (McCartney *et al.* 2003). Basically, there are two common methods for the detection of products in real-time PCR: first, non-specific fluorescent dyes that intercalate with any double-stranded DNA; and second, sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter which permits detection only after hybridisation of the probe with its complementary sequence. Therefore, these alternative detection chemistries make real-time PCR more suitable for multiplexing detection purposes. Regardless of the chemistry used, generated signals are frequently measured by accompanying software which gives data normalisation and a number of automatic options of analysis. At present, a variety of competing real-time PCR instruments with multiplexing and high throughput applications have been proposed through different companies.

Detection based on non-specific label method

SYBR Green dye. In real-time PCR assays, DNA binding dyes are utilised as fluorescent reporters to

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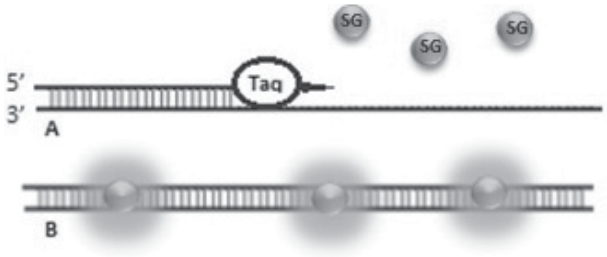

Figure 1. Diagram of a SYBR Green dye. When SYBR Green binds unspecifically to double-stranded DNA, it is able to emit green light as fluorescence. The amount of fluorescence is directly proportional to the amount of PCR products amplified (SG – SYBR Green dye)

monitor the reaction. As the PCR product accumulates with each consecutive cycle of amplification, the fluorescence of reporter dye is enhanced. Therefore, it is possible to monitor the PCR reaction during the exponential phase by recording the amount of fluorescence emission at each cycle. So, during real-time PCR, if the increase in fluorescence of the reporter dye is plotted against the log of the corresponding amount of template, a linear relationship is observed. SYBR Green is an intercalating dye which binds to a minor groove of the double-stranded DNA and is the dye most widely used for real-time PCR assays. SYBR Green does not emit fluorescence in its free form, emitting the fluorescence signal only when binding to the dsDNA (Figure 1).

Since unmodified oligonucleotide primers or no labelled oligonucleotides can be used with SYBR Green, its application is cheaper than other detection forms. However, the principal drawback to intercalation based detection of product accumulation is that specific and nonspecific products produce signal. So, the formation of non-specific amplicons can lead to false positive results in the quantification (Giulietti *et al.* 2001). To develop as much information from real-time PCR based on SYBR Green, the reaction should be followed by melting curve analysis in which melting temperature (T_m) of generated product is determined. The shape of the melting curve and the determined melting temperature depend on the PCR product concentration, its size and nucleotide base composition (Dreo *et al*. 2012). The SYBR Green sensitivity has been improved by the use of an anti-*Taq* antibody, which reduces the nonspecific product generation (Morrison *et al.* 1998). Besides, YO-PRO-1 (Ishiguro *et al*. 1995) and also ethidium bromide can also be used as intercalating dye but carcinogenic nature renders its use limiting (Schaad *et al.* 2003).

Detection based on sequence specific methods

TaqMan probe based detection. TaqMan probes are dual-labelled hydrolysis probes and utilise the 5' exonuclease activity of the *Taq* DNA polymerase for measuring the amount of target sequences. TaqMan probes consist of a sequence of 25–30 nucleotides in length which is labelled with a donor fluorophore (as reporter) at the 5' end, and an acceptor dye (as quencher) at the 3' end (Figure 2). Generally, a fluorophore is a molecule that absorbs light energy and is promoted to an excited state, and a quencher is a molecule that can receive energy from a fluorophore and disperse the energy by proximal quenching or by fluorescence resonance energy transfer (FRET) (Didenko 2001). In FRET quenching, as a dynamic quenching mechanism, the fluorophore transfers its energy to the quencher, and the energy is released as light of a longer wavelength (Schena *et al.* 2013). So, until the time when the probe is not hydrolysed, the quencher and the fluorophore remain in proximity to each other, separated by the probe length. However, this proximity does not entirely quench the fluorescence of the reporter dye and a background fluorescence is detected (Didenko 2001). During PCR, the probe hybridizes to the single-stranded DNA (ssDNA) template. In the extension step, the probe cleaves by the 5'-nuclease activity of *Taq* DNA polymerase when the enzyme reaches the probe, resulting in the separation of the fluorescent reporter dye from the quencher, thus generating a fluorescent signal (Figure 2). The fluorescence intensity is therefore a direct consequence of the amplification process.

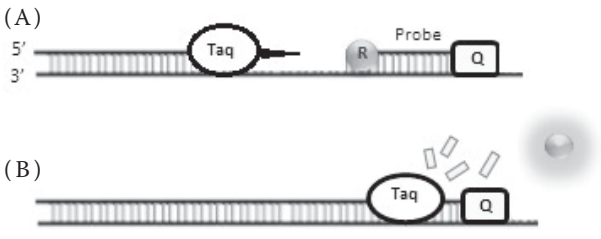

Figure 2. Diagram of TaqMan probe. The TaqMan probe binds to the target DNA, and the primer binds as well. Because the primer is bound, *Taq* DNA polymerase can create a complementary strand (A). The reporter dye is released from the extending double-stranded DNA created by the *Taq* DNA polymerase. Away from the quenching dye, the light emitted from the reporter dye in an excited state can be observed (B) (Q – quencher dye; R – fluorescent reporter dye)

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Unlike FRET quenching, in proximal quenching, while the probe is intact, the quencher absorbs the energy from the reporter dye due to close proximity between them and dissipates as heat (Schena *et al.* 2004). As a result, no fluorescence is discerned. However, in TaqMan, the fluorophore is quenched by FRET. There are several fluorophores and quenchers that can be paired; fluorophores such as TET, JOE, HEX, FAM, ROX, and TAMRA and, quenchers such as Methyl Red, TAMRA, and DABCYL are commonly used for TaqMan assays (Wittwer *et al.* 2001). So, one advantage of the TaqMan probe over SYBR Green dye is that specific hybridisation between probe and target DNA sequence is required to produce fluorescent signal. A TaqMan real-time PCR assay can be also multiplexed, because it can amplify and detect several distinct sequences in a single PCR reaction tube due to possibility of labelling of the fluorogenic probes with different detectable reporter dyes (Schena *et al*. 2004), avoiding cross similarities with primers and probes in multiplex reactions. However, the labelling of TaqMan probe with double dyes and its designing is more complicated than in SYBR Green primers, making this assay more expensive than SYBR Green assay (Okubara *et al.* 2005).

Molecular beacon based detection. Molecular beacons are single-stranded oligonucleotide hybridization probes that form a stem and loop (hairpin) structure. The loop of the probe is complementary to the target sequence, and its two ends are also complementary to each other. A fluorophore is tagged at the 5' end of the probe and a quencher at the 3' end (Figure 3). When the probe sequence in the loop anneals to a complementary nucleic acid target sequence, the stem portion of the beacon separates out and hybridises to the target, resulting in the fluorescence emission. Fluorophores such as FAM, TAMRA, TET, and ROX and a quenching dye, typically DABCYL, are the most commonly used. In the absence of a complementary target sequence, the beacon remains closed and there is no appreciable fluorescence (Figure 3). Fluorescence is screened during each annealing step when the beacon is attached to its complementary target. So, the amount of fluorescence at each cycle depends on the amount of specific product.

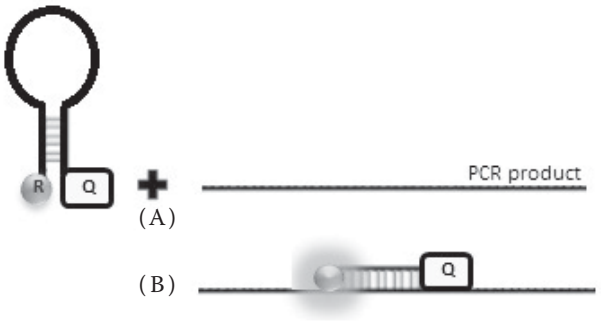

Figure 3. Diagram of molecular beacon. Detection of PCR product by molecular beacon. When the beacon binds to the PCR product, it is able to fluoresce when excited by the appropriate wavelength of light. The amount of fluorescence is directly proportional to the amount of PCR product amplified (Q – quencher dye; R – fluorescent reporter dye)

Unlike TaqMan probe, fluorescence quenching is proximal, due to the close contact of fluorophore and quencher that is more efficient than FRET-based quenching (Schena *et al.* 2004). Also, in comparison with linear probes, molecular beacons are especially suitable for identifying point mutations, because the hairpin-like structure makes mismatched hybrids thermally less stable than hybrids between the corresponding linear probes and their mismatched target (Giulietti *et al.* 2001). Furthermore, quenching of molecular beacons through a direct transfer of energy from the fluorophore to quencher is possible. So, a common quencher molecule can be used, increasing the number of fluorophores that can simply be used as reporters (Mhlanga & Malmberg 2001). These properties of molecular beacons can be used to develop extremely specific assays that other types of probes could not achieve. However, the design of molecular beacon is more difficult than other types of probes.

Scorpion probe based detection. Scorpion primers are bi-functional molecules in which a primer is covalently linked to a specific probe sequence that is held in a hairpin–loop form with a fluorophore at one end and a quencher at the other. At the 5' end, the Scorpion primer sequence contains a non-target sequence as PCR blocker at the start of the hairpin loop that prevents polymerase read-through. This structure brings the fluorophore in close proximity with the quencher and avoids fluorescence. Scorpion makes the molecular beacon technique more efficient by combining the functions of the probe and the 5' PCR primer (Didenko 2001). In the absence of the target, the quencher nearly absorbs the fluorescence emitted by the fluorophore. As soon as annealing between the primer–probe and the target occurs, scorpion primer combines to the PCR product and then the probe sequence in the tail curls back to hybridise with the sequence of target. As the tail of the scorpion and the amplicon are part of the same

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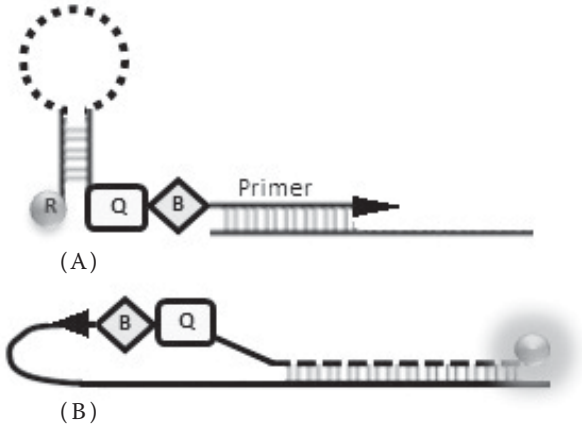

Figure 4. Diagram of Scorpion probe. During annealing, the hairpin primer binds to the template, and is then extended (A). During subsequent denaturation, the reporter separates from the quencher, and the loop sequence binds to the internal target sequence (B) (Q – quencher dye; R – fluorescent reporter dye; B – blocker)

strand of DNA, the interaction is intra-molecular (Giulietti *et al.* 2001; Arya *et al.* 2005). Hybridisation reaction unties the hairpin loop, separating the fluorophore and the quencher, which leads to an increase in the fluorescence emitted (Figure 4).

Similar to molecular beacons, in Scorpion-PCR fluorescence quenching is proximal, because of the close contact of fluorophore and quencher (Schena *et al*. 2004). Scorpion primers can also be used to identify point mutations by using multiple probes. Each probe can be tagged with a different fluorophore to produce different colours. In Scorpion primers, the probe is physically coupled to the primer which means that the reaction leading to signal generation is a unimolecular which is efficiently instantaneous (Tomlinson *et al.* 2007). So, this leads to stronger signals, more reliable probe design, enhanced discrimination, and shorter reaction times compared with molecular beacons and TaqMan probes. Relative sensitivities of the real-time PCR chemistries in increasing order are: SYBR Green I < TaqMan < Molecular beacons < Scorpion (Okubara *et al*. 2005). In fact, one should bear in mind that probe-based chemistries reveal a greater dynamic range than SYBR Green chemistry.

Real-time PCR considerations

DNA extraction. A critical pre-analysis step for real-time PCR assays is DNA extraction. Since many extraction methods can result in DNA revealing different levels of purity and final yield, the quality of the final results can be significantly affected (Olexova *et al.* 2004). Therefore, the main purpose of DNA extraction is providing a good quality of DNA with a low concentration of substances inhibiting PCR reactions for subsequent analyses. Furthermore, DNA extraction protocols should provide comparable results with samples differing widely in physical and chemical composition, organic content, microbial populations, etc. (Schena *et al*. 2013). Basically, substances such as polysaccharides, phenolic and humic compounds in soil and plant must be removed (Tsai & Olson 1991). The inhibitory substances can be removed using different columns and resins such as gel filtration (also known as size exclusion) resins, agarose gel electrophoresis, template dilution (Miller 2001), and commercial kits. Several practical DNA extraction methods such as isopropanol, silica-columns, magnetic beads, lyophilisation, freeze-grind and heat treatment have been used for extraction of high-quality DNA from microorganisms such as fungi and bacteria in order to minimise the influence of the extraction in the quantification of a low copy number of target (Cullen & Hirsch 1998; Reeleder *et al*. 2003; Ippolito *et al*. 2004; Weller *et al*. 2007; Garrido *et al.* 2009; Williams *et al*. 2009; Bilodeau *et al*. 2012). Also, to extract DNA from soil samples, a variety of extraction kits are available. Unlike dilution plating method that requires culturing of organisms for enumeration, a number of commercial kits are available to extract RNA or DNA from plant tissues. Totally, simplicity and rapidity as well as the absence of harmful chemical compounds are the main advantages of commercial kits. Combination of an efficient extraction method with a real-time PCR-based technique provides a useful and rapid tool for determining of pathogens populations and other organisms.

Target genes selection. Another crucial step in real-time PCR assays is the identification of appropriate target DNA regions. Sequences of the target primer must be unique to identify sequences of the target in the sample of interest with high specificity and efficiency so that to recognise virulence genes or a particular organism. The ribosomal DNA genes (rDNA) provide efficient targets because they have conserved and variable sequences that allow highly sensitive detection. But, due to its universal nature, the level of discrimination lies at the species levels (Schaad *et al*. 2003). Among the variable regions, the

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kinternal transcribed spacer (ITS) within prokaryotic and eukaryotic rDNA operons is the most widely sequenced in phytopathogens. Also, intergenic spacer (IGS) sequences are difficult for amplification and sequencing, but they can be more variable than the ITS sequences. Thus, they are exploited to design diagnostic assays when there are not enough differences available across the ITS (Schena *et al*. 2004). Typically, IGS regions of bacterial 16S ribosomal RNA genes, ITS regions of the fungal ribosomal RNA genes and mitochondrial small subunit rDNA have been used most commonly for PCR-based identification of plant pathogens. These multicopy sequences contain sufficient sequence diversity at the species or subspecies levels (Okubara *et al*. 2005). Moreover, the *β-tubulin* gene has been used for diagnosis purposes of plant pathogens when variation of ITS sequence is not appropriate for production of a taxon-specific diagnostic (Schena *et al*. 2004). Generally, genotypic differences in elongation factor 1 alpha (*EF1-α*), random-amplified polymorphic DNA/sequence-characterised amplified region (RAPD/SCAR)-based targets, and other single- or low-copy sequences have proven suitable for real-time PCR assays (Okubara *et al*. 2005).

Primer and probe design. Primer design is aimed at obtaining a balance between two goals: efficiency and specificity of amplification. Efficiency can be viewed as the proportion of templates that are used to synthesise new strands with each round of PCR. So, the most important issue for designing efficient PCR primers is that they must bind to the target site efficiently under PCR conditions. Specificity can generally be defined as the tendency for a primer to hybridise to its intended target and not to nonspecific targets and so primers that only amplify one product will provide the best assay sensitivity (Hyndman & Mitsuhashi 2003). Typically, 2–3 bases are sufficient to produce highly specific primer and probe using average stringent amplification conditions and should be preferentially localised close to the 3' end of the sequence (Fredslund & Lange 2007; Schena *et al.* 2013). Primers designed for use in cPCR can also be applied in real-time PCR tests if amplicon size criteria are met. However, amplicon sizes frequently used for cPCR are very long to support the design of efficient real-time PCR assays (Montes-Borrego *et al*. 2011). Specific primers and probes can be properly designed for SYBR Green, TaqMan probes, Molecular beacons and Scorpion PCR assays using primer design software such as Primer Express (PE Applied Biosystems, USA), Primer3 (Whitehead Institute, USA), Clustal X (Version 2.0 or greater) and Beacon designer (PREMIER Biosoft International, USA). After primers and probes are designed, their specificity should be checked by in silico analyses using the Basic Local Alignment Search Tool (BLAST) in GenBank to confirm the existence of similar sequences.

Further considerations. Real-time PCR assays may give false negative results for environmental samples due to several reasons, including the low number of targets, degradation of the target DNA by nucleases or reagent problems. Besides, as mentioned above, the problem with PCR inhibitors is frequent when environmental samples are assessed. For detecting inhibitor effects, causing false negative, an internal positive control such as the amplification of a housekeeping gene or a conserved DNA segment can be included in the assays (Schena *et al*. 2013). Another problem is the risk of nucleic acids contamination by external sources, such as exogenous DNA from cultures or from previous experiments while the PCR amplification products accumulate by repeated amplification of the same target sequence (Okubara *et al*. 2005). The risk of contamination can be reduced by precise activities such as using negative control, positive control, and reagent control in each PCR run.

Detection of plant pathogens

The most influential characteristic of real-time PCR is its suitability for quantitative analyses. In recent years, real-time PCR as a valuable and versatile tool has been used with accuracy and high throughput quantification of specific target in most agricultural fields such as plant protection and plant biotechnology. Also, simultaneous detection of more than one organism provides significant benefits particularly for diagnostic programs dealing with a lot of samples using real-time PCR (Cooke *et al*. 2007). Although in multiplex real-time PCR assays several target DNAs can be simultaneously detected from different microorganisms by differences in emission wavelength or amplicon size, a major limitation of multiplexing is the competition between different primers and probes, resulting in lower specificity and sensitivity (Okubara *et al.* 2005). However, the detection of more than two targets without reduction of sensitivity has also been reported (Schena *et al*. 2006). In order to obtain the best results, primer

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and probe design, amplification conditions, and amplicon length should be optimised. As the first available real-time chemistry, SYBR Green I can be used in a wide range of plant pathogens, whereas the TaqMan chemistry has an inherently higher degree of specificity, reliability, and performance, as discussed in the previous sections. The specific hybridization between probe and target DNA that is required to generate a fluorescent signal is the main advantage of fluorogenic probes over DNA binding dyes (Okubara *et al*. 2005; Garrido *et al*. 2009). A search of relevant published articles indicates that the relative frequency of chemistries applied in real-time PCR technique to date is, in increasing order: Molecular beacons < Scorpion < SYBR Green I < TaqMan (Schena *et al*. 2013). Generally, sequence specific methods guarantee higher specificity levels which are particularly important when they are used for detection and quantification of pathogens in natural samples or within symptomless tissues (Schena *et al*. 2004). In the following sections, some uses of real-time PCR techniques applied for routine detection/quantification of plant pathogens in agricultural systems are shown.

Viruses and viroids. Detection of plant viruses can be directly accomplished, although a reverse transcription (RT) step is essential prior to PCR amplification to generate the complementary DNA (cDNA). In fact, real-time RT-PCR has an additional cycle of reverse transcription that leads to formation of a DNA molecule from a RNA molecule. This is done because RNA is less stable as compared to DNA (Ingle & Kushner 1996). Real-time RT-PCR method based on TaqMan chemistry was reliably used to detect reproducibly of 1000 molecules of the target transcript (as little as 500 fg total RNA) of *Tomato spotted wilt virus* (TSWV) in infected tomato plants and was more sensitive (10-folds) than the conventional RT-PCR (Roberts *et al*. 2000). In another study, real-time RT-PCR method based on scorpion probe using specific primers designed to a highly conserved coat protein (CP) region was effectively used to detect *Grapevine fan leaf virus* (GFLV) in the nematode vector *Xiphinema index* collected from the rhizosphere of grapevine plants (Finetti-Sialer & Ciancio 2005). Altogether, the most widely used real-time RT-PCR protocols are based on two different chemistries including the fluorescent dye SYBR Green and TaqMan probe. These methods have proven successful in detecting and identifying different viruses on different hosts (Table 1). Since the viruses may be deactivated quickly when the infected plants dry up under field conditions, few studies have been done to detect the

Table 1. Examples of real-time RT-PCR assays for detection of plant pathogenic viruses and viroids (last 6 years)

Pathogen	Variant of chemistry	Host plant/ Specimen	Reference
Citrus exocortis viroid (CEVd), *Hop stunt viroid* (HSVd)	multiplex TaqMan	citrus, plum	Papayiannis (2014)
Tobacco etch virus (TEV)	SYBR Green	irrigation water	Chen *et al*. (2014)
Apple chlorotic leaf spot virus (ACLSV), *Cherry green ring mottle virus* (CGRMV)	multiplex SYBR Green	peach	Zhao *et al*. (2013)
Grapevine fanleaf virus (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine fleck virus* (GFkV), *Grapevine leafroll associated virus* 1,3 (GLRaV-1,3)	multiplex TaqMan	grapevine	Lopez-Fabuel *et al*. (2013)
Rice black streaked dwarf virus (RBSDV), *Southern rice black streaked dwarf virus* (SRBSDV)	multiplex TaqMan	rice	Zhang *et al*. (2013)
Tobacco etch virus (TEV), *Potato virus* Y (PVY), *Tobacco vein banding mosaic virus* (TVBMV)	multiplex TaqMan	tobacco	Dai *et al*. (2013)
Rice tungro bacilliform virus (RTBV), *Rice tungro spherical virus* (RTSV)	SYBR Green	rice	Sharma & Dasgupta (2012)
Tobacco mosaic virus (TMV)	SYBR Green	soil	Yang *et al*. (2012)
Peach latent mosaic viroid (PLMVd)	SYBR Green	peach	Parisi *et al*. (2011)
Peach latent mosaic viroid (PLMVd)	TaqMan	peach	Luigi & Faggioli (2011)
Wheat dwarf virus (WDV)	TaqMan	wheat, insect vectors	Zhang *et al*. (2010)
Citrus viroid III (CVd-III)	SYBR Green	citrus	Rizza *et al*. (2009)

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plant viruses in soil and water (Boben *et al*. 2007; Yang *et al*. 2012).

Viroids represent a group of extremely primitive pathogenic entities consisting exclusively of nucleic acids that are capable of independent replication and inducing diseases when introduced into susceptible plant cells (Narayanasamy 2011). Because of the absence of a protein component that is present in the viruses, diagnostic approaches based upon serology have not been applicable for the detection of viroids. So, PCR-based techniques such as RT-PCR and real-time RT-PCR are the most reliable and sensitive tests for detection of viroids in infected plants (Boonham *et al.* 2004). In this respect, a real-time RT-PCR assay based on TaqMan chemistry was developed by Boonham *et al.* (2004) in order to detect *Potato spindle tuber viroid* (PSTVd), a quarantine pathogen in Europe, which was 1000 times more sensitive compared with a chemiluminescent assay. Furthermore, real-time RT-PCR assay based on the SYBR-Green I was developed for the quantitative detection of *Citrus exocortis viroid* (CEVd) and *Citrus viroid*-IIb (CVd-IIb) causing citrus exocortis and citrus cachexia diseases in symptomless host citrus plants. Primer pairs designed from highly conserved regions of the genome of different variants of each viroid amplified DNA fragments of 83-bp (CEVd) and 133-bp (CVd-II), which were detected by the increasing fluorescence observed during the reaction. The evidence indicated that real-time RT-PCR is to be a useful tool for fast and reliable diagnosis of citrus viroids (Tessitori *et al.* 2005). A few number of real-time RT-PCR procedures are available for detection of a variety of plant viroids in plant materials (Table 1).

Bacteria and phytoplasmas. Control of diseases caused by plant pathogenic bacteria frequently needs precise detection, followed by suitable identification of the causal organism (Palacio-Bielsa *et al.* 2011). Several real-time PCR systems have been validated with significant improvements in speed, specificity, and sensitivity for detection and direct quantification of plant pathogenic bacteria in different environmental samples. For instance, a SYBR Green I real-time PCR assay was developed for specific detection and quantification of *Xanthomonas axonopodis* pv. *citri*, causing Type A citrus canker, and *X. axonopodis* pv. *aurantifolii*, causing Type B and C citrus canker, based on primers designed to short fragments from highly conserved regions (*pthA* gene), as it represents a diagnostic indicator for citrus canker-causing xanthomonads. The assay enabled reliable detection as low as 1 pg of total *X. citri* DNA isolated from diseased leaf lesions (Mavrodieva *et al*. 2004). Also, a TaqMan real-time PCR assay was developed for the reliable detection of *Xylella*

Table 2. Examples of real-time PCR assays for detection of plant pathogenic bacteria and phytoplasmas (last 6 years)

Pathogen	Variant of chemistry	Host plant/Specimen	Reference
Gluconacetobacter diazotrophicus	SYBR Green	sugarcane	Boa-Sorte *et al*. (2014)
Leifsonia xyli subsp. *xyli*	TaqMan	sugarcane	Pelosi *et al*. (2013)
Erwinia amylovora	SYBR Green, TaqMan	apple, pear	Kaluzna *et al*. (2013)
Clavibacter michiganensis subsp. *michiganensis*	TaqMan	tomato	Johnson & Walcott (2012)
Candidatus Phytoplasma mali, *Ca*. *P. prunorum*, *Ca*. P. pyri	TaqMan	apple, pear *Prunus* species, insect vectors	Mehle *et al*. (2012)
Candidatus Phytoplasma mali	TaqMan	apple, insect vectors	Baric (2012)
Xanthomonas arboricola pv. *pruni*	TaqMan	*Prunus* species	Palacio-Bielsa *et al*. (2011)
Pseudomonas syringae pathovars: *syringae, tomato, maculicola, tabaci, atropurpurea, phaseolicola, pisi*, and *glycinea*	TaqMan	tomato, plum, crucifer, tobacco, brome grass, bean, pea, soybean	Xu & Tambong (2011)
Pectobacterium carotovorum subsp. *carotovorum*, *P. chrysanthemi*	SYBR Green	konnyaku potato, soil	Wu *et al*. (2011)
Xylella fastidiosa subsp. *fastidiosa*, *Xylella fastidiosa* subsp. *multiplex*	TaqMan	grapevine, almond, apple, oak, insect vectors	Harper *et al*. (2010)
Pseudomonas syringae pv. *phaseolicola*	TaqMan	bean	Cho *et al*. (2010)
Candidatus Phytoplasma isolates	TaqMan	*Catharanthus roseus*, coconut	Hodgetts *et al*. (2009)

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fastidiosa (Xf) strains at low concentrations of the bacterium in almonds, grapes, and insect vectors with a high degree of sensitivity and specificity using primers designed to a unique region common to the sequenced genomes of four Xf strains associated with Pierce's disease in grapes, oleander leaf scorch, almond leaf scorch, and citrus variegated chlorosis. Actually, no amplicons were obtained with non-Xf bacterial strains (Francis *et al*. 2006). In addition, an increasing number of real-time PCR procedures are available for detection and quantification of bacteria in plant materials, although TaqMan probes are the most commonly used ones (Table 2). The lack of growth in pure culture, uneven distribution in the phloem of the infected plant, and low concentration (especially in woody hosts) are most important obstacles for efficient diagnosis of phytoplasmas, that means their quantification can only be achieved in the presence of high levels of host DNA (Galetto *et al*. 2005; Weintraub & Jones 2010). So, a few studies have been done to detect the pathogenic phytoplasmas in plants (Table 2).

Fungi and oomycetes. Fungi and oomycetes include the most important plant pathogens and their accurate detection is an important part of preventive disease management strategies. Among the PCR-based techniques, real-time PCR has been proven to be simple and reliable for detection and quantification of these pathogens. For instance, *Phytophthora fragariae* var. *fragariae* causing root rot disease is a quarantine organism and is present in most European countries. So, a real-time PCR assay using TaqMan probe and Molecular beacon was utilised as a sensitive and reliable detection test. With Molecular beacon the pathogen was detected in a quantitative order similarly to TaqMan probe, which were able to detect levels as low as 1 fg DNA of the target pathogen present in plant tissues. In this study, the sensitivity of Molecular beacon and TaqMan probes against a dilution series of *P. fragariae* genomic DNA was equivalent (Bonants *et al.* 2004). Also, Bilodeau *et al*. (2007) compared three different chemistries including SYBR Green, TaqMan, and Molecular beacons using sequences of β-tubulin, ITS and elicitin gene regions for detection of *P. ramorum* causing sudden oak death. The results showed that all three real-time PCR assays could separate the pathogen from 65 other species of *Phytophthora* in all infected samples. However, TaqMan real-time PCR assay based on ITS and elicitin regions was shown to be more sensitive than others in detecting and differentiating *P. ramorum*.

Detection of seed-borne fungal pathogens is an imperative part of seed health testing programs. In this regard, the presence of *Tilletia caries*, causing common bunt disease, the most important seed-borne

Table 3. Examples of real-time PCR assays for detection of plant pathogenic fungi and oomycetes

Pathogen	Variant of chemistry	Host plant/Specimen	Reference
Ceratocystis coerulescens C. polonica, C. laricicola, C. fujiensis	SYBR Green, TaqMan	*Pinaceae, Eucalyptus* sp., insect vectors	Lamarche *et al.* (2014)
Phytophthora infestans	SYBR Green	potato	Hussain *et al.* (2014)
Pythium aphanidermatum, P. helicoides, P. myriotylum	TaqMan	tomato	Li *et al.* (2014)
Sclerotinia sclerotiorum	SYBR Green	carrot, bean, lettuce, onion, peach	Parker *et al.* (2014)
Magnaporthe oryzae	TaqMan	rice	Su'udi *et al.* (2013)
F. oxysporum f.sp. *melonis*	SYBR Green	melon	Haegi *et al.* (2013)
Phytophthora infestans	TaqMan	potato	Clement *et al.* (2013)
Verticillium dahliae	TaqMan	strawberry	Bilodeau *et al.* (2012)
Botrytis cinerea	SYBR Green	grape	Diguta *et al.* (2010)
Fusarium virguliforme	TaqMan	soybean	Mbofung *et al.* (2011)
Colletotrichum acutatum, C. gloeosporioides	TaqMan	strawberry	Garrido *et al.* (2009)
Puccinia graminis, P. striiformis, P. triticina, P. recondita f.sp. *secalis*	TaqMan	cereals and grasses	Barnes & Szabo (2007)
Phytophthora nicotianae, P. citrophthora	Scorpion	citrus	Ippolito *et al.* (2004)
Rosellinia necatrix	Scorpion	fruit and forest tree species	Schena & Ippolito (2003)

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disease of wheat, was detected and also the level of contamination in apical meristems of different wheat varieties was quantified using SYBR Green I real-time PCR procedure based on primers designed to IGS region of the rDNA. The assay could quantify pathogen mycelium in wheat varieties in the range from 0.34 ng to 15 µg per one growing tip. Therefore, this method can be applied in the screening process for bunt resistance in wheat as well as in certification and breeding processes at early stages of plant development (Zouhar *et al*. 2010). On the other hand, some plant pathogenic fungi are also vectors of plant viruses like *Polymyxa* spp. Several viruses such as *Beet necrotic yellow vein virus* (BNYVV), *Barley yellow mosaic virus* (BaYMV), and *Soil-borne wheat mosaic virus* (SBWMV) are transmitted by *Polymyxa* spp. Accordingly, *P. betae* and *P. graminis* were directly detected and quantified from as little as 500 mg of infested soils using TaqMan real-time PCR based on primers and probes designed to ITS regions (Ward *et al*. 2004). Generally, a variety of real-time PCR techniques for detection and quantification of numerous important plant pathogenic fungi and oomycetes has been described (Table 3).

CONCLUDING REMARKS

Real-time PCR has a significant potential in quantifying low disease levels with high sensitivity and speed that was inconceivable in plant pathology a few years ago. The technique is extremely promising in order to quantify pathogen populations, whereas other PCR-based techniques qualify only for the identification/detection of the microbial communities. With accurate optimisation, real-time PCR can provide specific, reliable, and high throughput detection and quantification of target DNA in various environmental samples in real time, which is not achievable with other PCR-based methods. In fact, real-time PCR is an ideal technique to measure levels of inoculum threshold, which has a positive impact on epidemiological studies, and for evaluating the efficacy of methodologies used to prevent distribution of the pathogens into non-infected agricultural fields. As knowledge regarding individual microorganisms' genomes increases, the use of this technique for a broad range of microorganisms will undoubtedly increase. In addition, this growing list of applications suggests that real-time PCR will be an increasingly preferred method in the future, opening new research opportunities associated with a comprehensive understanding of ecology and population dynamics of pathogens with the final intent of optimising plant disease management strategies.

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References

Acero F.J., Carbu M., El-Akhal M.R., Garrido C., Gonzalez-Rodriguez V.E., Cantoral J.M. (2011): Development of proteomics-based fungicides: New strategies for environmentally friendly control of fungal plant diseases. International Journal of Molecular Sciences, 12: 795–816.

Arya M., Shergill I.S., Williamson M., Gommersal L., Arya N., Patel H.R. (2005): Basic principles of real-time quantitative PCR. Expert Review of Molecular Diagnostics, 5: 209–219.

Baric S. (2012): Quantitative real-time PCR analysis of '*Candidatus* Phytoplasma mali' without external standard curves. Erwerbs-Obstbau, 54: 147–153.

Barnes C.W., Szabo L.J. (2007): Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. Phytopathology, 97: 717–727.

Bilodeau G.J., Levesque C.A., Decock A.W.A.M., Duchaine C., Briere S., Uribe P., Martin F.N., Hamelin R.C. (2007): Molecular detection of *Phytophthora ramorum* by real-time polymerase chain reaction using TaqMan, SYBR Green, and Molecular beacons. Phytopathology, 97: 632–642.

Bilodeau G.J., Koike S.T., Uribe P., Martin F.N. (2012): Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. Phytopathology, 102: 331–343.

Boa-Sorte P.M.F., Simoes-Araujo J.L., de Melo L.H.V., de Souza Galisa P., Leal L., Baldani J.I., Baldani V.L.D. (2014): Development of a real-time PCR assay for the detection and quantification of *Gluconacetobacter diazotrophicus* in sugarcane grown under field conditions. African Journal of Microbiology Research, 8(31): 2937–2946.

Boben J., Kramberger P., Petrovic N., Cankar K., Peterka M., Strancar A., Ravnikar M. (2007): Detection and quantification of tomato mosaic virus in irrigation waters. European Journal of Plant Pathology, 118: 59–71.

Bonants P.J.M, van Gent-Pelzer M.P.E., Hooftman R., Cooke D., Guy D.C., Duncan J.M. (2004): A combination of baiting and different PCR formats, including measurement

doi: 10.17221/104/2014-PPS

of real-time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. European Journal of Plant Pathology, 110: 689–702.

Boonham N., Perez L.G., Mendez M.S., Peralta E.L., Blockley A., Walsh K., Barker I., Mumford R.A. (2004): Development of a real-time RT-PCR assay for the detection of *Potato spindle tuber viroid*. Journal of Virological Methods, 116: 139–146.

Capote N., Pastrana A.M., Aguado A., Torres P.S. (2012): Molecular tools for detection of plant pathogenic fungi and fungicide resistance. In: Cumagun C.J. (ed.): Agricultural and Biological Sciences "Plant Pathology". Rijeka, InTech: 151–202.

Cooke D.E.L., Schena L., Cacciola S.O. (2007): Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. Journal of Plant Pathology, 89: 13–28.

Chen W., Dai J., Zhang H., Jiao H., Cheng J. Wu Y. (2014): Concentration and detection of tobacco etch virus from irrigation water using real-time PCR. Turkish Journal of Agriculture and Forestry, 38: 471–477.

Cho M.S., Jeon Y.H., Kang M.J., Ahn H.I., Baek H.J., Na Y.W., Choi Y.M., Kim T.S. Park D.S. (2010): Sensitive and specific detection of phaseolotoxigenic and nontoxigenic strains of *Pseudomonas syringae* pv. *phaseolicola* by TaqMan real-time PCR using site-specific recombinase gene sequences. Microbiological Research, 165: 565–572.

Clement J.A.J., Baldwin T.K., Magalon H., Glais I., Gracianne C., Andrivon D., Jacquot E. (2013): Specific detection and quantification of virulent/avirulent *Phytophthora infestans* isolates using a real-time PCR assay that targets polymorphisms of the *Avr3a* gene. Letters in Applied Microbiology, 56: 322–332.

Cullen D.W., Hirsch, P.R. (1998): Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biology and Biochemistry, 30: 983–993.

Dai J., Peng H., Chen W., Cheng J., Wu Y. (2013): Development of multiplex real time PCR for simultaneous detection of three Potyviruses in tobacco plants. Journal of Applied Microbiology, 114: 502–508.

Didenko V.V. (2001): DNA probes using fluorescence resonance energy transfer (FRET): designs and applications. BioTechniques, 31: 1106–1121.

Diguta C.F., Rousseaux S., Weidmann S., Bretin N., Vincent B., Benatier M.G., Alexander H. (2010): Development of a qPCR assay for specific quantification of *Botrytis cinerea* on grapes. FEMS Microbiology Letter, 313: 81–87.

Dreo T., Pirc M., Ravnikar M. (2012): Real-time PCR, a method fit for detection and quantification of *Erwinia amylovora*. Trees, 26: 165–178.

Espy M.J., Uhl J.R., Sloan L.M., Buckwalter S.P., Jones M.F., Vetter E.A., Yao J.D.C., Wengenack N.L., Rosenblatt J.E., Cockerill F.R., Smith T.F. (2006): Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clinical Microbiology Reviews, 19: 165–256.

Finetti-Sialer M.M., Ciancio A. (2005): Isolate-specific detection of *Grapevine fanleaf virus* from *Xiphinema index* through DNA-based molecular probes. Phytopathology, 95: 262–268.

Francis M., Lin H., Rosa J.C, Doddapaneni H., Civerolo E.L. (2006): Genome-based PCR primers for specific and sensitive detection and quantification of *Xylella fastidiosa*. European Journal of Plant Pathology, 115: 203–213.

Fredslund J., Lange M. (2007): Primique: automatic design of specific PCR primers for each sequence in a family. BMC Bioinformatics, 8: 369.

Galetto L., Bosco D., Marzachi C. (2005): Universal and group-specific real-time PCR diagnosis of flavescence dorée (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. Annals of Applied Biology, 147: 191–201.

Garrido C., Carbu M., Acreo F.J., Boonham N., Coyler A., Cantoral J.M., Budge G. (2009): Development of protocols for detection of *Colletotrichum acutatum* and monitoring of strawberry anthracnose using real-time PCR. Plant Pathology, 58: 43–51.

Garrido C., Acero F.G.F., Carbu M., Rodriguez V.E.G., Liniero E., Cantoral J.M. (2012): Molecular microbiology applied to the study of phytopathogenic fungi. In: Magdeldin S. (ed.): Biochemistry, Genetics and Molecular Biology. Rijeka, InTech: 139–156.

Giulietti A., Overbergh L., Valckx D., Decallone B., Bouillon R., Mathieu C. (2001): An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods, 25: 386–401.

Goud J.C., Termorshuizen A.J. (2003): Quality of methods to quantify microsclerotia of *Verticillium dahliae* in soil. European Journal of Plant Pathology, 109: 523– 534.

Hadidi A., Levy L., Podleckis E.V. (1995): Polymerase chain reaction technology in plant pathology. In: Singh R.P., Singh U.S. (eds): Molecular Methods in Plant Pathology. London, CRC Press Inc.: 167–187.

Haegi A., Catalano V., Luongo L., Vitale S., Scotton M., Ficcadenti N., Belisario A. (2013): A newly developed real-time PCR assay for detection and quantification of *Fusarium oxysporum* and its use in compatible and incompatible interactions with grafted melon genotypes. Phytopathology, 103: 802–810.

Harper S.J., Ward L.I., Clover G.R.G. (2010): Development of LAMP and real-time PCR methods for the rapid detection of *Xylella fastidiosa* for quarantine and field applications. Phytopathology, 100: 1282–1289.

doi: 10.17221/104/2014-PPS

Hassain T., Singh B.P., Anwar F. (2014): A quantitative real-time PCR based method for the detection of *Phytophthora infestans* causing Late blight of potato, in infested soil. Saudi Journal of Biological Sciences, 21: 380–386.

Hodgetts J., Boonham N., Mumford R., Dickinson M. (2009): Panel of 23S rRNA gene-based real-time PCR assays for improved universal and group-specific detection of phytoplasmas. Applied and Environmental Biology, 75: 2945–2950.

Hyndman D.L., Mitsuhashi M. (2003): PCR primer design. In: Bartlett M.S., Stirling D. (eds): PCR Protocols. Series: Methods in Molecular Biology, Vol. 226. 2nd Ed. New York, Humana Press: 81–88.

Ingle C.A., Kushner S.R. (1996): Development of an *in vitro* mRNA decay system for *Escherichia coli*: poly(A) polymerase I is necessary to trigger degradation. Proceedings National Academy of Science USA, 93: 12926–12931.

Ippolito A., Schena L., Nigro F., Ligorio V.S., Yaseen T. (2004): Real-time detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil. European Journal of Plant Pathology, 110: 833–843.

Ishiguro T., Saitoh J., Yawata H., Yamagishi H., Iwasaki S., Mitoma Y. (1995): Homogeneous qualitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater. Analytical Biochemistry, 229: 207–213.

Johnson K.L., Walcott R.R. (2012): Progress towards a real-time PCR assay for the simultaneous detection of *Clavibacter michiganensis* subsp. *michiganensis* and *Pepino mosaic virus* in tomato seed. Journal of Phytopathology, 160: 353–363.

Kaluzna M., Pulawska J., Mikicinski A. (2013): Evaluation of methods for *Erwinia amylovora* detection. Journal of Horticultural Research, 21: 65–71.

Lamarche J., Stewart D., Pelletier G., Hamelin R.C., Tanguay P. (2014): Real-time PCR detection and discrimination of the *Ceratocystis coerulescens* complex and of the fungal species from the *Ceratocystis polonica* complex validated on pure cultures and bark beetle vectors. Canadian Journal of Forest Research, 44: 1103–1111.

Li M., Y. Ishiguro Y., Otsubo K., Suzuki H., Tsuji T., Miyake N., Nagai H., Suga H., Kageyama K. (2014): Monitoring by real-time PCR of three water-borne zoosporic *Pythium* species in potted flower and tomato greenhouses under hydroponic culture systems. European Journal of Plant Pathology, 140: 229–242.

Lievens B., Grauwet T.J.M.A., Cammue B.P.A., Thomma B.P.H.J. (2005): Recent developments in diagnostics of plant pathogens: a review. Recent Research Developments in Microbiology, 9: 57–79.

Lopez-Fabuel I., Wetzel T., Bertolini E., Bassler A., Vidal E., Torres L.B., Yuste A., Olmos A. (2013): Real-time multiplex RT-PCR for the simultaneous detection of the five main grapevine viruses. Journal of Virological Methods, 188: 21–24.

Luigi M., Faggioli F. (2011): Development of quantitative real-time RT-PCR for the detection and quantification of *Peach latent mosaic viroid*. European Journal of Plant Pathology, 130: 109–116.

Mahuku G.S., Platt H.W. (2002): Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. American Journal of Potato Research, 79: 107–117.

Martin R.R., James D., Levesque C.A. (2000): Impacts of molecular diagnostic technologies on plant disease management. Annual Review of Phytopathology, 38: 207–239.

Martin F.N., Tooley P.W., Blomquist C. (2004): Molecular detection of *Phytophthora ramorum*, the causal agent of sudden oak death in California, and two additional species commonly recovered from diseased plant material. Phytopathology, 94: 621–631.

Mavrodieva V., Levy L., Gabriel D.W. (2004): Improved sampling methods for real-time polymerase chain reaction diagnosis of citrus canker from field samples. Phytopathology, 94: 61–68.

Mbofung G.C.Y., Fessehaie A., Bhattacharyya M.K., Leandro L.F.S. (2011): A new TaqMan real-time polymerase chain reaction assay for quantification of *Fusarium virguliforme* in soil. Plant Disease, 95: 1420–1426.

McCartney H.A., Foster S.J., Fraaije B.A., Ward, E. (2003): Molecular diagnostics for fungal plant pathogens. Pest Management Science, 59: 129–142.

Mehle N., Nikolic P., Gruden K., Ravnikar M., Dermastia M. (2012): Real-time PCR for specific detection of three *Phytoplasmas* from the apple proliferation group. In: Dickinson M., Hodgetts J. (eds): Phytoplasma: Methods and Protocols. Series: Methods in Molecular Biology, Vol. 938. New York, Humana Press: 269–281.

Mhlanga M.M., Malmberg L. (2001): Using molecular beacons to detect single-nucleotide polymorphisms with real-time PCR. Methods, 25: 463–471.

Miller D.N. (2001): Evaluation of gel filtration resins for the removal of PCR-inhibitory substances from soils and sediments. Journal of Microbiological Methods, 44: 49–58.

Montes-Borrego M., Munoz-Ledesma F.J., Jimenez-Diaz R.M., Landa B.B. (2011): Real-time PCR quantification of *Peronospora arborescens*, the opium poppy downy mildew pathogen, in seed stocks and symptomless infected plants. Plant Disease, 95: 143–152.

Morrison T.B., Weis J.J., Wittwer C.T. (1998): Quantification of low-copy transcripts by continuous SYBR green I

doi: 10.17221/104/2014-PPS

monitoring during amplification. BioTechniques, 24: 954–962.

Narayanasamy P. (2011): Detection of Virus and Viroid Pathogens in Plants. In: Microbial Plant Pathogens-Detection and Disease Diagnosis. Viral and Viroid Pathogens, Vol. 3. Dordrecht, Springer: 7–220.

Okubara P.A., Schroeder K.L., Paulitz T.C. (2005): Real-time polymerase chain reaction: applications to studies on soilborne pathogens. Canadian Journal of Plant Pathology, 27: 300–313.

Olexova L., Dovicovicova L., Kuchta T. (2004): Comparison of three types of methods for the isolation of DNA from flours, biscuits and instant paps. European Food Research and Technology, 218: 390–393.

Palacio-Bielsa A., Cubero J., Cambra M.A., Collados R., Berruete I.M., Lopez M.M. (2011): Development of an efficient real-time quantitative PCR protocol for detection of *Xanthomonas arboricola* pv. *prun*i in *Prunus* species. Applied and Environmental Microbiology, 77: 89–97.

Papayiannis L.C. (2014): Diagnostic real-time RT-PCR for the simultaneous detection of *Citrus exocortis viroid* and *Hop stunt viroid*. Journal of Virological Methods, 196: 93–9.

Parisi O., Lepoivre P., Jijakli M.H. (2011): Development of a quick quantitative real-time PCR for the *in vivo* detection and quantification of *Peach latent mosaic viroid*. Plant Disease, 95: 137–142.

Parker M.L., McDonald M.R., Boland G.J. (2014): Evaluation of air sampling and detection methods to quantify airborne ascospores of *Sclerotinia sclerotiorum*. Plant Disease, 98: 32–42.

Pelosi C.S., Lourenco M.V., Silva M., Santos A.Z., Franca S.C., Marins M. (2013): Development of a TaqMan real-time PCR assay for detection of *Leifsonia xyli* subsp *xyli*. Tropical Plant Pathology, 38: 343–345.

Reeleder R.D., Capell B.B., Tomlinson L.D., Hickey W.J. (2003): The extraction of fungal DNA from multiple large soil samples. Canadian Journal of Plant Pathology, 25: 182–191.

Rizza S., Nobile G., Tessitori M., Catara A., Conte E. (2009): Real time RT-PCR assay for quantitative detection of *Citrus viroid* III in plant tissues. Plant Pathology, 58: 181–185.

Roberts C.A., Dietzgen R.G., Heelan L.A., Maclean D.J. (2000): Real-time RT-PCR fluorescent detection of tomato spotted wilt virus. Journal of Virology Methods, 88: 1–8.

Schaad N.W., Frederick R.D., Shaw J., Schneider W.L., Hickson R., Petrillo M.D., Luster D.G. (2003): Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Annual Review of Phytopathology, 41: 305–324.

Schena L., Ippolito A. (2003): Rapid and sensitive detection of *Rosellinia necatrix* in roots and soils by real time Scorpion-PCR. Journal of Plant Pathology, 85: 15–25.

Schena L., Nigro F., Ippolito A., 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.

Schena L., Hughes K.J.D., Cooke D.E.L. (2006): Detection and quantification of *Phytophthora ramorum*, *P. kernoviae, P. citricola and P. quercina* in symptomatic leaves by multiplex real-time PCR. Molecular Plant Pathology, 7: 365–379.

Schena L., Li Destri Nicosia M.G., Sanzani S.M., Faedda R., Ippolito A., Cacciola S.O. (2013): Development of quantitative PCR detection methods for phytopathogenic fungi and oomycetes. Journal of Plant Pathology, 95: 7–24.

Sharma S., Dasgupta I. (2012): Development of SYBR Green I based real-time PCR assays for quantitative detection of *Rice tungro bacilliform virus* and *Rice tungro spherical virus*. Journal of Virological Methods, 181: 86–92.

Su'udi M., Kim J., Park J.M., Bae S.C., Kim D., Kim Y.H., Ahn I.P. (2013): Quantification of rice blast disease progressions through TaqMan real-time PCR. Molecular Biotechnology, 55: 43–48.

Tessitori M., Rizza S., Reina A., Catara V. (2005): Real-time RT-PCR based on Sybr-Green I for the detection of citrus exocortis and citrus cachexia disease. In: Hilf M.E., Duran-Vila N., Rocha-Peña M.A. (eds): Proceedings 16th Conference of the International Organization of Citrus Virologists, Nov 3–6, 2004. Riverside, USA: 456–459.

Tomlinson J.A., Baker I., Boonham N. (2007): Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. Applied and Environmental Microbiology, 73: 4040–4047.

Tsai Y.L., Olson B.H. (1991): Rapid method for direct extraction of DNA from soil and sediments. Applied and Environmental Microbiology, 57: 1070–1074.

Ward L.I., Beales P.A., Barnes A.V., Lane C.R. (2004): A real-time PCR assay based method for routine diagnosis of *Spongospora subterranea* on potato tubers. Journal of Phytopathology, 152: 633–638.

Weintraub P.G., Jones P. (2010): Phytoplasmas: genomes, plant hosts and vectors. Plant Pathology, 59: 1177–1178.

Weller S.A., Beresford-Jones N.J., Hall J., Thwaites R., Parkinson N., Elphinstone J.G. (2007): Detection of *Xanthomonas fragariae* and presumptive detection of *Xanthomonas arboricola* pv. *fragariae*, from strawberry leaves, by real-time PCR. Journal of Microbiological Methods, 70: 379–383.

Williams N., Hardy G.E.St.J., O'Brien P.A. (2009): Analysis of the distribution *Phytophthora cinnamomi* in soil at a disease site in Western Australia using nested PCR. Forest Pathology, 39: 95–109.

doi: 10.17221/104/2014-PPS

Wittwer C.T., Herrman M.G., Gundry C.N., Elenitoba-Johnson K.S.J. (2001): Real-time multiplex PCR assays. Methods, 25: 430–442.

Wu J., Diao Y., Gu Y., Hu Z. (2011): Molecular detection of *Pectobacterium* species causing soft rot of *Amorphophallus konjac*. World Journal of Microbiology and Biotechnology, 27: 613–618.

Xu R., Tambong J.T. (2011): A TaqMan real-time PCR assay targeting the cytochrome o ubiquinol oxidase subunit II gene for detection of several pathovars of *Pseudomonas syringae*. Canadian Journal of Plant Pathology, 33: 318–331.

Yang J.G., Wang F.L., Chen D.X., Shen L.L., Qian Y.M., Liang Z.Y., Zhou W.C., Yan T.H. (2012): Development of a one-step immunocapture real-time RT-PCR assay for detection of *Tobacco mosaic virus* in soil. Sensors, 12: 16685–16694.

Zhang X., Zhou G., Wang X. (2010): Detection of *Wheat dwarf virus* (WDV) in wheat and vector leafhopper (*Psammotettix alienus* Dahlb.) by real-time PCR. Journal of Virological Methods, 169: 416–419.

Zhang P., Mar T.T., Liu W.W., Li L., Wang X.F. (2013): Simultaneous detection and differentiation of *Rice black streaked dwarf virus* (RBSDV) and *Southern rice black streaked dwarf virus* (SRBSDV) by duplex real time RT-PCR. Virology Journal, 10: 24.

Zhao Z., Yu Y., Zhang Z., Liang P., Ma Y., Li S., Wang H. (2013): A duplex, SYBR Green I-based RT-qPCR assay for the simultaneous detection of *Apple chlorotic leaf spot virus* and *Cherry green ring mottle virus* in peach. Virology Journal, 10: 255.

Zouhar M., Mazáková J., Prokinová E., Váňová M., Ryšánek P. (2010): Quantification of *Tilletia caries* and *Tilletia controversa* mycelium in wheat apical meristem by real-time PCR. Plant Protection Science, 46: 107–115.

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