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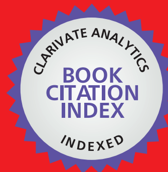


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# Molecular Tools for Detection of Plant Pathogenic Fungi and Fungicide Resistance

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## 1. Introduction

Plant pathogenic fungi are the causal agents of the most detrimental diseases in plants, including economically important crops, provoking considerable yield losses worldwide. Fungal pathogens can infect a wide range of plant species or be restricted to one or few host species. Some of them are obligate parasites requiring the presence of the living host to grow and reproduce, but most of them are saprophytic and can survive without the presence of the living plant, in the soil, water or air. Isolates of a fungal species can be differentiated by morphological characteristics, host range (*formae speciales*), pathogenic aggressiveness (pathotypes or races) or their ability to form stable vegetative heterokaryons by fusion between genetically different strains (belonging to the same vegetative compatibility group, VCG).

Detection and accurate identification of plant pathogens is one of the most important strategies for controlling plant diseases to initiate preventive or curative measures. Special interest should be taken in the early detection of pathogens in seeds, mother plants and propagative plant material to avoid the introduction and further spreading of new pathogens in a growing area where it is not present yet. For that reason, the availability of fast, sensitive and accurate methods for detection and identification of fungal pathogens is increasingly necessary to improve disease control decision making. Traditionally, the most prevalent techniques used to identify plant pathogens relied upon culture-based morphological approaches. These methods, however, are often time-consuming, laborious, and require extensive knowledge of classical taxonomy. Other limitations include the difficulty of some species to be cultured *in vitro*, and the inability to accurately quantify the pathogen (Goud & Termorshuizen, 2003). These limitations have led to the development of molecular approaches with improved accuracy and reliability. A high variety of molecular methods have been used to detect, identify and quantify a long list of plant pathogenic fungi. Molecular methods have also been applied to the study of the genetic variability of pathogen populations, and even for the description of new fungal species. In general, these methods are much faster, more specific, more sensitive, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. Additionally, these techniques allow the detection and identification of non-culturable

microorganisms, and due to its high degree of specificity, molecular techniques can distinguish closely related organisms at different taxonomic levels. Here, we review the most important tools for molecular detection of plant pathogenic fungi, their applicability, and their implementation in horticultural and agricultural practices.

On the other hand, once the pathogenic fungus is already established in a given crop growing area, the use of synthetic fungicides constitutes the main strategy to control plant diseases, since these compounds act quickly and effectively. The disadvantages of continued use of fungicides are their limited spectrum and the emergence of resistant fungal isolates. This fact leads to many yield losses as control systems are not longer effective. The development of resistance to fungicides in fungal pathogens and the growing public concern over the health and environmental hazards associated with the high level of pesticide have resulted in a significant interest in knowing more about fungal resistance. The emergence of more stringent regulations regarding pesticide residues means that one of the main priorities is to ensure food security by reducing the use of fungi toxics. For this reason, it is important to identify and characterize the mechanisms involved in the emergence of strains resistant to fungicides used for control diseases and to know the molecular methods currently available to detect them.

## 2. Molecular methods for detection of plant pathogenic fungi

### 2.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is the most important and sensitive technique presently available for the detection of plant pathogens. PCR allows the amplification of millions of copies of specific DNA sequences by repeated cycles of denaturation, polymerisation and elongation at different temperatures using specific oligonucleotides (primers), deoxyribonucleotide triphosphates (dNTPs) and a thermostable *Taq* DNA polymerase in the adequate buffer (Mullis & Faloona, 1987). The amplified DNA fragments are visualized by electrophoresis in agarose gel stained with EtBr, SYBR Green or other safer molecule able to intercalate in the double stranded DNA, or alternatively by colorimetric (Mutasa et al., 1996) or fluorometric assays (Fraaije et al., 1999). The presence of a specific DNA band of the expected size indicates the presence of the target pathogen in the sample. Advances in PCR-based methods, such as real-time PCR, allow fast, accurate detection and quantification of plant pathogens in an automated reaction. Main advantages of PCR techniques include high sensitivity, specificity and reliability. Moreover, it is not necessary to isolate the pathogen from the infected material reducing the diagnosis time from weeks to hours, and allowing the detection and identification of non-culturable pathogens. This characteristic has been especially useful in the analysis of symptomless plants. However, the frequent presence of PCR inhibitors in the plant tissues or soil can reduce considerably the sensitivity of the reaction and even result in false negative detection. Many attempts have been carried out to overcome this issue (see below). Another disadvantage of the PCR methodology is the occurrence of false positive results due to the presence of DNA or PCR products (amplicons) contaminants. For this reason, it is advisable to separate physically and temporally pre- and post-PCR analysis. Another failure of PCR-based methods is the inability to discriminate viable from non-viable fungi or fungal structures, which might inform of the real threat for the plant. Development of a prior PCR step involving enrichment culturing (BIO-PCR) (Ozakman &

Schaad, 2003) or amplification of fungal RNA (RT-PCR) (Lee et al., 1989) may solve this problem to same extent. Like this, many PCR variants have been developed to improve sensitivity, specificity, rapidity and throughput, and to allow the quantification of the fungal pathogen in the plant and the environment.

### 2.1.1 Starting material

Collection and preparation of samples is a critical step for the detection of plant pathogenic fungi. The starting material may be symptomatic plant tissue (roots, leaves, stems, flowers, fruits or seeds), soil, water or air. Also, latent infections can be detected on symptomless plants.

In the case of infected plants, the first step consists in the cultivation of the fungi. After surface sterilisation of the plant tissue (e.g. with 1% sodium hypochlorite or 50% hydrogen peroxide) small pieces are transferred to Petri dishes containing an appropriate nutrient medium (e.g. Potato Dextrosa Agar, PDA) supplemented with antibiotics to prevent bacterial contaminants (usually streptomycin), and incubated at required temperature (25-30ºC) for pathogen development. If the fungal pathogen has infected deep internal tissues, cutting the plant material to expose core tissues may be necessary. Pure fungal colonies must be obtained either by isolating single spore or single hyphal tips. In the first case, a spore suspension of the fungus is prepared and serial dilutions in sterile water are obtained. An optimal dilution is transferred to Petri dishes containing 2% agar-water and incubated at appropriate temperature to induce spore germination. A single germinating spore is then isolated and a pure colony of the fungus is obtained. To get a single hyphal tip, a small segment of fungal growth is transferred to a new Petri dish containing nutrient medium and incubated at optimum temperature to allow growth of the mycellium. A separate hyphal edge is them transferred to new agar plate to obtain a pure colony of the fungal species. Isolation of the fungus from an infected plant tissue can however obviate the presence in the infected plant of other non-culturable pathogens or fungi having special culture conditions. Competition between distinct fungi can also mask the detection by culturing of an infecting pathogen. For DNA extraction, fungal mycelium must be firstly homogenized in a mortar in the presence of liquid nitrogen. Other homogenisation procedures obviate the use of liquid nitrogen, e.g, grinding the mycellium inside centrifuge tubes with the help of sealed tips or plastic pistils that fit perfectly into the tubes coupled to household drills (González et al., 2008).

Alternatively, total DNA from the plant and the fungi can be isolated together from the infected plant tissue. That allows skipping the fungi culture step, although DNA from different fungal species or strains may be obtained. Homogenisation of the infected plant tissues is usually performed in the presence of liquid nitrogen. Then, an extraction buffer is added to obtain a crude extract. Alternatively, plant material may be introduced into individual plastic bags containing a soft net in the case of tender material (Homex, Bioreba; Stomacher, AES Laboratoire) or a heavy net in the case of dry or harder material such as bark tissue or seeds (PlantPrint Diagnostics). Homogenisation can be made by the help of a manual roller or by the use of special apparatus designed to facilitate the homogenisation (e.g. Homex, Bioreba). As in the case of fungal mycellium, small amounts of plant tissue can be homogenised inside centrifuge tubes using pistils coupled to electric drills in the presence of extraction buffers.

When analysing soil samples, the main focus for phytopathologists is the isolation of DNA from different microorganisms and then, the specific detection and monitoring of the fungus(i) of interest. Classical approach consists of cultivating the soil fungi in different media and screen for the desired pathogen. However, many microorganisms from the soil community can not be isolated by this procedure. An alternative method is to isolate DNA directly from the soil sample without prior culturing. Protocols using enzymatic (e.g. protease, chitinase, glucanase) or mechanical lysis (glass-beads beating, freeze-thawing, vigorous shaking, microwave or grinding in liquid nitrogen) have been reported, but a combination of both procedures seems to be the more effective (Anderson I. C. & Parkin, 2007, Jiang et al., 2011). In the same way, improved protocols for an efficient isolation of DNA from water for detection and monitoring of plant pathogens have been reported (Pereira et al., 2010).

During the homogenisation process, polysaccharides and phenolic compounds from plants or humic and fulvic acids from soils can be released that can inhibit the *Taq* DNA polymerase leading to the occurrence of false negatives (Munford et al., 2006; Tebbe & Vahjen, 1993; Wilson, 1997). This problem may be partially overcome by the use in the extraction buffer of some compounds such as polyvinylpyrrolidone (PVP) or cetyltrimethyl ammonium bromide (CTAB) for plant extracts, and bovine sero albumine (BSA) for soil samples (Anderson I.C. & Cairney, 2004), or by removing inhibitors by the use of spin/vacuum columns. Some PCR variants such as Magnetic Capture-Hybridisation PCR have been developed to remove the presence of PCR inhibitors in plant extracts (see below). In addition, it is increasingly common to use an internal positive control of the PCR reaction either by the amplification of a conserved plant gene (e.g. cytochrome oxidase I, *cox* I) in multiplex (Bilodeau, et al., 2009) or in a parallel assay (Garrido et al., 2009), or by the addition of an exogenous DNA and their corresponding primers to each reaction (Cruz-Pérez et al., 2001).

### 2.1.2 DNA extraction methods

There are no universally validated nucleic-acids extraction protocols for fungi, infected plant material or soil. Many published protocols are available to ensure an efficient and reproducible method for DNA extraction from plants (revised by Demeke and Jenkins, 2010; Biswas and Biswas, 2011); from fungi (Chi et al., 2009; Feng et al., 2010; González-Mendoza et al., 2010; Niu et al., 2008; Zelaya-Molina et al., 2011; Zhang, Y. J. et al., 2010); and from soil (revised by Hirsch et al., 2010).

The use of commercial kits for nucleic acids extraction, either general or specifically designed for plant material, fungi or soil is gaining acceptance because they are easy to use and are enable to efficiently remove inhibitory compounds during the purification process. They are generally based on magnetic beads or spin columns, although quicker protocols are also available (e.g. QuickExtract™ Plant DNA Extraction Solution, Epicentre). Automated or semi-automated systems have also been developed to allow the isolation of nucleic acids from different samples, among others, QIAxtractor, QIAgen; 6700 Automated Nucleic Acid Workstation, Life Technologies; Magna Pure LC extraction system, Roche; Solucion m2000, Abbott.

Purification of nucleic acids is labour intensive, costly, time-consuming and not applicable when a large number of samples need to be analysed. Several attempts have been undertaken

to avoid the nucleic acids isolation step. One of them uses few microliters of crude extract loaded and immobilized on small pieces of paper, e.g FTA cards. A subsequent lysis of the cells in appropriate buffer allows the release of nucleic acids that are fixed in the membrane and protected from degradation. DNA can be stored on dry cards for several years in a dry place at room temperature without decreasing the sensitivity of detection (Smith & Burgoyne, 2004). Moreover, membrane immobilised-DNA is suitable of transportation or mail to other laboratories. Suzuki et al. (2006) reported that nucleic acids recovered from FTA cards could be used for the detection of *Aspergillus oryzae*, releasing the DNA from the fungal tissue by treatment in a microwave oven before application to the membranes. Grund et al. (2010) used FTA cards coupled to PCR for the detection of plant pathogens including oomycetes such as *Phytophthora* and filamentous fungi such as *Fusarium*.

#### 2.1.3 Selection of target DNA to amplify

Generally, conserved known genes with enough sequence variation are selected for designing PCR diagnostic assays and performing phylogenetic analysis. The most common region used for these purposes has been the internal transcribed spacer (ITS) region of ribosomal RNA genes. rDNA region consists of multiple copies (up to 200 copies per haploid genome) arranged in tandem repeats comprising the 18S small subunit, the 5.8S, and the 28S large subunit genes separated by internal transcribed spacer regions (ITS1 and ITS2) (Bruns et al., 1991; Liew et al., 1998). This region contains highly conserved areas adequate for genera- o species-consensus primer designing (RNA ribosomal genes), alternate with highly variable areas that allow discrimination over a wide range of taxonomic levels (ITS region) (White et al., 1990). The ITS region is ubiquitous in nature and found in all eukaryotes. In addition, the high copy numbers of rRNA genes in the fungal genome enable a highly sensitive PCR amplification. Furthermore, a large numbers of ribosomal sequences are publicly available in databases, facilitating the validation and the reliability of the detection assays.

Traditionally, molecular identification of plant pathogenic fungi is accomplished by PCR amplification of ITS region followed by either restriction analysis (Durán et al., 2010) or direct sequencing and BLAST searching against GenBank or other databases (White et al., 1990). Identification could be a challenge when using BLAST analysis with ITS sequences because there can be minimal or no differences between some species or, in some cases, intraspecific variation can confuse the boundaries between species (e.g., *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* have identical ITS sequences). The sequence analysis of the ITS region has additionally served to proposed new species. Abad et al. (2008) aligned ITS regions of *Phytophthora* spp. associated with root rot from different geographic origins and hosts with GenBank sequences from other *Phytophthora* species and proposed a new isolate *Phytophthora bisheria* sp. nov. Just as, Burgess et al. (2009) also distinguished new and undescribed taxa of *Phytophthora* from natural ecosystems. This alignment analysis has also been used for the identification of new species of *Pythium* (de Cock & Lévesque, 2004; Nechwatal & Mendgen, 2006; Paul, 2006, 2009; Paul et al., 2005; Paul & Bala, 2008).

The ITS region has also been widely used in fungal taxonomy and it is known to show variation between species e.g., between *Phytium ultimum* and *P. helicoides* (Kageyama et al., 2007); *Peronospora arborescens* and *P. cristata* (Landa et al., 2007); *Colletotrichum gloeosporioides* and *C. acutatum* (Kim J. T. et al., 2008), and within species e.g allowing differentiation of

*Puccinia striiformis* f. sp. *tritici* (Zhao et al., 2007), and distinguishing between weakly and high virulent isolates of *Leptosphaeria maculans* (Xue et al., 1992), or difining anastomosis subgroups of *Rhizoctonia solani* (Budge, 2009; Godoy-Lutz et al., 2008).

The ITS region remains an important locus for molecular identification of fungi. However, as more sequence data is collected from a wider range of fungal isolates, the utility of alternative loci for accurate species identification is increasing. The intergenic spacer sequence (IGS) placed between the 28S and 18S rRNA genes is the region with the greatest amount of sequence variation in rDNA. It is frequently used in PCR-based methods when there are not enough differences available across the ITS. Primers in this region have been designed to detect and identify *Verticillium dahliae* and *V. alboatrum* (Schena et al., 2004) and to distinguish pathogenic and non-pathogenic *Fusarium oxysporum* in tomato (Validov et al., 2011). As another example, Inami et al. (2010) differentiated *Fusarium oxysporum f. sp lycopersici*, and its races using primers and TaqMan-MGB probes based on IGS and avirulent SIX genes.

Other housekeeping genes with higher variability are being more extensively used to develop diagnostics for fungi, including nuclear genes such as β-tubulin (Aroca et al., 2008; Fraaije et al., 2001; Mostert et al., 2006), translation elongation factor 1 alpha (*TEF* 1α) (Geiser et al., 2004; Knutsen et al., 2004, Kristensen et al., 2005), calmodulin (Mulè et al., 2004), avirulence genes (Lievens et al., 2009), and mitochondrial genes such as the multicopy *cox* I and *cox* II and their intergenic region (Martin & Tooley, 2003; Nguyen & Seifert, 2008; Seifert et al., 2007). Mating type genes also show high diversity and fast evolutionary rate and could be used for inter- and intra-species differentiation, e.g. Foster et al. (2002) distinguished between the two mating types of *Pyrenopeziza brassicae*. Moreover, Martínez-Espinoza et al. (2003) used mating type genes to specifically detect *Ustilago maydis* in maize cultivars. To enhance the specificity of a diagnostic assay, a combination of multiple diagnostic regions is recommended. Many authors have followed this multi-locus diagnostic strategy, e.g. Collado-Romero et al. (2008) studied the evolutionary relationships among *Verticillium dahliae* vegetative compatibility groups by AFLP fingerprints and sequence analysis of actin, β-tubulin, calmodulin, and histone 3 genes, the ITS region, and a *V. dahliae*-specific sequence; Dixon et al. (2009) demonstrated the host specialisation and phylogenetic diversity of *Corynespora cassiicola* using the ITS region, actine gene and two random hypervariable loci; Glienke et al. (2011) performed sequence analysis of the ITS region and partial *TEF* 1α, actin and glyceraldehyde-3-phosphate dehydrogenase (GPDH) genes to study the genetic diversity of *Phyllosticta* spp. allowing differentiation of pathogenic and non pathogenic species and describing two new *Phyllosticta* species; Inderbitzin et al. (2010) verified a high species diversity in *Botryosphaeriaceae* species performing phylogenetic analyses based on six loci, including the ITS region and *TEF* 1α, GPDH, a heat shock protein, histone-3 and β–tubulin genes; Shimomoto et al. (2011) used RAPDs and sequences from β-tubulin, *TEF* 1α, calmodulin and actin genes to detect pathogenic and genetic variation among isolates of *Corynespora cassiicola*.

### 2.1.4 Single nucleotide polymorphisms (SNPs)

Closely related pathogens showing different host ranges or pathogenicity often differ in only a single to a few base pairs in target genes commonly used for identification. Therefore, the ability to discriminate single nucleotide polymorphisms (SNPs) should be pursued in any diagnostic assay. Based on the DNA nucleotide sequence difference in the

mitochondrial *cox* I gene, a SNP method has been developed to detect and differentiate isolates of *Phytophthora ramorum* from Europe and those originating in the United States (Kroon et al., 2004). In other experiment, polymorphisms detected in the microsatellite flanking regions of *Phytophthora infestans* allowed the development of a SNP genetic marker system for typing this pathogen (Abbott et al., 2010). Many of the examples described below take advantage of SNPs for designing highly specific detection assays.

#### 2.1.5 Design of primers and probes

PCR methods are based on the use of specific oligonucleotides or primers that specifically hybridise with the DNA target and that are required to initiate the synthesis of the new DNA chain. In some real-time PCR methods additional specific oligonucleotides are used, named probes, that hybridise with the target DNA between the two primers. The design of primers and probes is crucial for PCR to be specific and efficient. Primer specificity relies on its sequence, length and GC content, which determines its melting temperature ($T_m$, the temperature at which 50% of primer-target duplex are hybridized). DNA amplified fragments (amplicons) size must be short enough to ensure efficiency of the reaction and high sensitivity (Singh and Singh, 1997). Real-time PCR usually requires shorter amplicons than conventional PCR. Balancing of primers concentration is necessary especially in multiplex PCR reactions and in real-time PCR using non-specific fluorescent dyes. Also, formation of hairpin structures or complementarities between primers must be avoided.

The first step for designing primers and probes consists in the aligment of the sequences of interest by the blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997) using sequences from the GenBank, EMBL and DDBJ databases. Partial or complete nucleotide sequences of many fungal genes are available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Genbank/) (Bethesda, MD, USA). Consensus sequences are used to design primers for detection of members of a same genus or species. If the consensus is not possible, degenerate primers can be used, although this may severely affect the overall sensitivity of the PCR reaction. On the other hand, variable sequences are useful for the differentiation of pathogens at lower taxonomic levels and for the analysis of the molecular variability of fungal population in phylogenetic studies.

Software packages for primer and probe design are available, among others Primer Express, Applied Biosystems; LightCycler Probe Design, Roche; Primer Explorer, Eiken Chemical Co.; Beacon Designer, Premier Biosoft International; Primer Premier, Premier Biosoft; Primer Analysis Software, OLIGO; Oligo perfect designer, Life Technologies/Invitrogen; or Primer3 (http://frodo.wi.mit.edu/primer3/).

#### 2.1.6 PCR-based methods

##### 2.1.6.1 Conventional PCR

Identification of fungal pathogens by conventional PCR may be achieved at different taxonomic levels (genus, species or strain) depending on the specificity of the primers. As recent examples, PCR methods for identification of *Sclerotium rolfsii* (Jeeva et al., 2010) and *Colletotrichum capsici* (Torres-Calzada et al., 2011) have been developed based in specific sequences of the ITS region. Improved variants of PCR have emerged with higher

sensitivity, specificity and throughput and allowing the quantification of fungi in infected plants or environment.

**2.1.6.2 Nested-PCR and Cooperational-PCR (Co-PCR)**

Nested PCR approach is used when an improvement of the sensitivity and/or specificity of detection is necessary. This method consists in two consecutive rounds of amplification in which two external primers amplify a large amplicon that is then used as a target for a second round of amplification using two internal primers (Porter-Jordan et al., 1990). The two reactions are usually performed in separated tubes involving time and effort and increasing the risks of false positives due to cross contamination. However, some improvements in the relative concentrations of the external and internal primers have permitted to perform the two reactions in a single closed tube supporting high throughput. This method has been widely used for detection and/or further characterisation of numerous fungi (Aroca & Raposo, 2007; Grote et al., 2002; Hong et al., 2010; Ippolito et al., 2002; Langrell et al., 2008; Meng and Wang 2010; Mercado-Blanco et al., 2001; Qin et al., 2011; Wu et al., 2011).

An alternative PCR method that enhances sensitivity and minimise contamination risks is the Co-operational PCR. In Co-PCR a single reaction containing the four primers, one pair internal to other, enhances the production of the longest fragment by the co-operational action of all amplicons (Olmos et al., 2002). Co-PCR is usually coupled with dot blot hybridisation by using a specific probe to enhance the specificity of the detection and provide a sensitivity level similar to nested PCR method. Martos et al. (2011) used this method for sensitive and specific detection of grapevine fungi. In both nested- and Co-PCR methods, the use of external primers can be used for generic amplification and the internal primers for further and more specific characterisation of the amplified product at species or strain level.

**2.1.6.3 Multiplex PCR**

Multiplex PCR is based on the use of several PCR primers in the same reaction allowing the simultaneous and sensitive detection of different DNA targets, reducing time and cost. This method is useful in plant pathology since plants are usually infected by more than one pathogen. Different fragments specific to the target fungi were simultaneously amplified and identified on the basis of their molecular sizes on agarose gels. Although the efficiency of amplification is strongly influenced by amplicon size (shorter amplicons may be amplified preferentially over longer ones), an accurate and careful design of primers and the optimisation of their relative concentrations are required to overcome this drawback and get an equilibrate detection of all target fungi. Multiplex PCR technique has been used for the simultaneous detection and differentiation of *Podosphaera xanthii* and *Golovinomyces cichoracearum* in sunflower (Chen et al., 2008); for detecting *Phytophthora lateralis* in cedar trees and water samples, including detection of an internal control in the same reaction (Winton & Hansen, 2001); for determining the mating type of the pathogens *Tapesia yallundae* and *T. acuformis* (Dyer et al., 2001); for differentiating two pathotypes of *Verticilliun albo-atrum* infecting hop (Radišek et al., 2004) and for distinguishing among eleven taxons of wood decay fungi infecting hardwood trees (Guglielmo et al., 2007). Due to the complexity of the design this technique has recently been replaced by other multiplexing techniques including multiplex real-time PCR (see below).

Another multiplexing method that allows simultaneous detection and identification of multiple oomycetes and fungi in complex plant or environmental samples is the use of ligation detection (LD) system using padlock probes (PLPs). PLPs are long oligonucleotide probes containing asymmetric target complementary regions at their 5' and 3' ends. Padlock probes also incorporate a desthiobiotin moiety for specific capture and release, an internal endonuclease IV cleavage site for linearization, and a unique sequence identifier, the so-called ZipCode, for standardised microarray hybridisation. DNA samples are PCR amplified and subjected to PLP ligation. Under perfectly hybridisation with the target, the PLPs are circularized by enzymatic ligation. Then, the probes are captured with streptavidin-coupled magnetic beads, cut at the internal cleavage site, allowing only the originally ligated PLPs to be visualized by hybridisation on a universal complementary ZipCode microarray. Padlock probes have been used for the simultaneous detection of *Phytophthora cactorum*, *P. nicotianae*, *Pythium ultimum*, *P. aphanidermatum*, *P. undulatum*, *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *F. solani*, *Myrothecium roridum*, *M. verrucaria*, *Verticillium dahliae* and *V. alboatrum* in samples collected from horticultural water circulation systems in a single assay (van Doorn et al., 2009).

#### 2.1.6.4 Magnetic Capture-Hybridisation (MCH)-PCR

This technique was developed to circumvent the presence of PCR inhibitors in plant extracts. Magnetic beads are coated with a biotinilated oligonucleotide that is specific of a DNA region of the fungus of interest. Hybridisation takes place between the fungal DNA and magnetic beads-oligomer and the conjugate is recovery separated from inhibitory compounds. After the magnetic capture-hybridisation, PCR amplification was carried out using species-specific primers. Langrell & Barbara, (2001) used this method to detect *Nectria galligena* in apple and pear trees.

#### 2.1.6.5 PCR-ELISA

This serological-based PCR method uses forward and reverse primers carrying at their 5′ end biotin and an antigenic group (e.g. fluorescein), respectively (Landgraf et al., 1991). PCR amplified DNA can be immobilized on avidin or streptavidin-coated microtiter plates via the biotin moiety of the forward primer and then can be quantified by an ELISA specific for the antigenic group of the reverse primer (e.g. anti-fluorescein antibody detected by colorimetric reactions). PCR-ELISA method is as sensitive as nested PCR. In addition, it does not require electrophoretic separation and/or hybridisation, and can be easily automated. All reactions can be performed in 96-well microtiter plates for mass screening of PCR products making them very suitable for routine diagnostic purposes. This procedure has been used for detection and differentiation of *Didymella bryoniae* from related *Phoma* species in cucurbits (Somai et al., 2002) and for detection of several species of *Phytophtora* and *Pythium* (Bailey et al., 2002).

#### 2.1.6.6 Reverse Transcription (RT)-PCR

An important limitation of molecular methods is the inability to distinguish living or dead fungi or fungal structures. So, results from detection and identification of fungal plant pathogens should be validated by pathogenicity tests. Since mRNA is degraded rapidly in dead cells, the detection of mRNA by RT–PCR is considered an accurate indicator of cell viability (Sheridan et al., 1998). In RT-PCR the RNA is reverse transcribed using the enzyme reverse transcriptase. The resulting cDNA is then amplified using conventional or any other

PCR-based method. RT-PCR has been used to detect viable populations of *Mycosphaerella graminicola* in wheat (Guo et al., 2005). A RT-nested-PCR method was applied for detection of *Oidium neolycopersici* in tomato (Matsuda et al., 2005). Even so, the most frequent application of this technique in phytopathology is the analysis of plant and fungal gene expression during disease development (Yang et al., 2010).

**2.1.6.7 *in situ* PCR**

This technique allows the amplification of specific gene sequences within intact cells or tissues combining two technologies: PCR and *in situ* hybridisation (ISH) (Long, 1993; Nuovo, 1992). The improved sensitivity of this technique allows the localization of one target copy per cell (Haase et al., 1990; Nuovo et al., 1991). However, background detection is usually high because nonspecific DNA synthesis during *in situ* PCR on tissue sections may occur (Nuovo et al., 1994). In addition, it is a time-consuming technique due to the need for a hybridisation step and technically demanding procedures such as light microscopy. Bindsley et al. (2002) used *in situ* PCR technique to identify *Blumeria graminis* spores and mycelia on barley leaves.

**2.1.6.8 PCR-DGGE**

This method is mainly applied for the analysis of the genetic diversity of microbial communities without the need of any prior knowledge of the species (Muyzer, 1999; Gothwal et al., 2007; Portillo et al., 2011). DGGE (Denaturing Gradient Gel Electrophoresis) and its variant TGGE (Temperature Gradient Gel Electrophoresis) use chemical gradient such as urea (DGGE) or temperature (TGGE) to denature and separate DNA samples when they are moving across an acrylamide gel. In PCR-DGGE target DNA from plant or environmental samples are firstly amplified by PCR and then subjected to denaturing electrophoresis. Sequence variants of particular fragments migrate at different positions in the denaturing gradient gel, allowing a very sensitive detection of polymorphisms in DNA sequences. In addition, PCR-DGGE primers contain a GC rich tail in their 5′ end to improve the detection of small variations (Myers et al., 1985). The bands obtained in the gel can be extracted, cloned or reamplified and sequenced for identification, being even possible to identify constituents that represent only 1% of the total microbial community. These techniques are very suitable for the identification of novel or unknown organisms and the most abundant species can be readily detected.

This method is however time-consuming, poorly reproducible and provides relative information about the abundance of detected species. Interpretation of the results may be difficult since the microheterogeneity present in some target genes may appear as multiple bands in the gel for a single species, leading to an overestimation of the community diversity. Furthermore, fragments with different sequences but similar melting behaviour are not always correctly separated. In other cases, the analysis of complex communities of microorganisms may result in blurred gels due to the large number of bands obtained.

A PCR-DGGE detection tool based in the amplification of the ITS region has been recently applied to detect multiple species of *Phytophthora* from plant material and environmental samples (Rytkönen et al., 2011). Other authors have used this technique to compare the structure of fungal communities growing in different conditions or environments, e.g. to study the impact of culture management such as biofumigation, chemifumigation or fertilisation on the relative abundance of soil fungal species (Omirou et al., 2011; Wakelin et al., 2008).

#### 2.1.6.9 Real-time PCR

Real-time PCR is currently considered 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 (Wittwer et al., 1997).

Many are the advantages of real time PCR over conventional PCR, including that this system does not require the use of post PCR processing (electrophoresis, colorimetric reaction or hybridisation), avoiding the risk of carryover contamination and reducing assay labour and material costs. In addition to its improved sensitivity and specificity, this technique allows the accurate quantification of the target pathogen, by interpolating the quantity measured to a standard curve with known amounts of target copies. This quantification characteristic is very useful in phytopathology in order to correlate the amount of fungus in a biological sample with the disease state, or to monitor the progress of the disease in an infected plant (Garrido et al., 2009). In addition, real-time PCR is a high throughput method for the analysis of a large number of samples due to the use of a plate-based system which permits the analysis of 96 or 384 samples at the same time. This characteristic facilitates the robotisation of the nucleic acids extraction and master mix preparation steps, reducing personal and gaining time. Portable real-time PCR machines are emerging as diagnostic tools for *on site* detection under field conditions providing a realistic option to perform molecular tests at the same place of the collection of samples (e.g. portable Cepheid SmartCycler). This is especially interesting in situations where a rapid diagnostic test is needed. Another advantage of real-time PCR is the capability to perform multiplex detection of two or more pathogens in the same reaction (see below).

Real-time RT-PCR chemistry can be based on the use of doubled-stranded DNA binding dyes, such as SYBR Green, specific fluorescent labelled probes such as TaqMan, Molecular Beacons, or Scorpions, or dye-primer based systems, such as hairpin primers or Plexor system.

*a. Non-specific fluorescent dyes*

SYBR Green I is a fluorescence intercalating dye with a high affinity for double-stranded DNA. The overall fluorescent signal from a reaction is proportional to the amount of double-stranded DNA (dsDNA) present in the sample, and increase as the target is amplified. The main advantage of the use of intercalator dyes is that no probe is required, which reduces assay setup and running costs. Binding dyes are also attractive because protocols using established primers and PCR conditions can readily be converted to the real-time method. However, intercalating dyes detect accumulation of both specific and non-specific PCR products. So, to assess the specificity of the reaction, it is necessary a further step of melting curve analysis that allows the identification of the PCR product by its $T_m$. Additionally, a fine optimisation of primers concentration is crucial to avoid formation of also detected primer-dimers. Quantification of targets using SYBR Green is not very accurate, since the amount of fluorescent signal is proportional to the mass of dsDNA produced in the reaction (amplification of a longer product will generate more signal than a shorter one). Generally, small amplicons must be selected (between 50 and 200 bp) for optimal efficiency. SYBR Green real-time PCR with melting curve analysis has been described as a simple, rapid, and reliable technique for the detection and identification of phytopathogenic fungi even in multiplex assays (Tabla 1).

| Pathogen | Real-time chemistry | Reference | Host plant |
|---|---|---|---|
| *Botrytis cinerea* | SYBR Green | Diguta el al., 2010 | Grape |
| *Cladosporium fulvum* | SYBR Green | Yan et al., 2008 | Tomato |
| *Colletotrichum acutatum* | SYBR Green | Samuelian et al., 2011 | Grape |
| *Fusarium avenaceum* | SYBR Green | Moradi et al., 2010 | Wheat |
| *Fusarium culmorum* | SYBR Green | Moradi et al., 2010 | Wheat |
| | SYBR Green multiplex | Brandfass & Karlovsky, 2006 | Cereals |
| *Fusarium graminearum* | SYBR Green | Moradi et al., 2010 | Wheat |
| | SYBR Green multiplex | Brandfass & Karlovsky, 2006 | Cereals |
| *Fusarium oxysporum* | SYBR Green | Jiménez-Fernández et al., 2010 | Chickpea |
| | | | Melon |
| | | | Pea |
| | | | Soil |
| *Fusarium oxysporum* f. sp. *vasinfectum* | SYBR Green | Abd-Elsalam et al., 2006 | Cotton |
| *Fusarium oxysporum* f. sp. *ciceris* | SYBR Green | Jiménez-Fernández et al., 2011 | Chickpea |
| | | | Soil |
| *Fusarium poae* | SYBR Green | Moradi et al., 2010 | Wheat |
| *Fusarium verticillioides* | SYBR Green | Kurtz et al., 2010 | Corn |
| *Greeneria uvicola* | SYBR Green | Samuelian et al., 2011 | Grape |
| *Macrophomina phaseolina* | SYBR Green | Babu et al., 2011 | Chickpea |
| | | | Soybean |
| | | | Pigeon pea |
| *Phoma sclerotioides* | SYBR Green | Larsen et al., 2007 | Alfalfa |
| | | | Wheat |
| *Phoma tracheiphila* | SYBR Green | Demontis et al., 2008 | Citrus |
| *Phytophthora capsici* | SYBR Green | Silvar et al., 2005 | Pepper |
| *Phytophthora cryptogea* | SYBR Green | Minerdi et al., 2008 | Gerbera |
| *Plasmodiophora brassicae* | SYBR Green | Sundelin et al., 2010 | Oilseed rape |
| *Puccinia horiana* | SYBR Green | Alaei et al., 2009 | Chrysanthemum |
| *Pythium irregular* | SYBR Green | Schroeder et al., 2006 | Wheat |
| | | | Barley |
| | | | Soil |
| *Pythium ultimum* | SYBR Green | Schroeder et al., 2006 | Wheat |
| | | | Barley |
| | | | Soil |
| *Rhizoctonia oryzae* | SYBR Green | Okubara et al., 2008 | Cereals |
| *Rhizoctonia solani* | SYBR Green | Okubara et al., 2008 | Cereals |
| *Rhynchosporium secalis* | SYBR Green | Fountaine et al. 2007 | Barley |
| *Sclerotinia sclerotiorum* | SYBR Green | Yin et al., 2009 | Oilseed rape |
| *Verticillium dahliae* | SYBR Green, Plexor | Attallah et al., 2007 | Potato |
| | SYBR Green | Gayoso et al., 2007 | Hot pepper |
| | SYBR Green | Markakis et al., 2009 | Olive |

Table 1. Examples of SYBR Green real-time PCR assays for detection of plant pathogenic fungi (last 6 years).

*b. Specific fluorescent labelled probes*

*b.1 Hydrolysis probes*

TaqMan chemistry (Heid et al., 1996) is based on the use of an oligonucleotide probe located between the two PCR primers and labelled with a fluorophore covalently attached to the 5′-end (reporter) and a quencher on the 3′end. When the reporter and the quencher are close, the emission of fluorescence is inhibited. After the PCR denaturation step, primers and probes specifically hybridise to the complementary target. The probe is then cleaved by the 5′-3′ exonuclease activity of the *Taq* DNA polymerase causing the separation of the fluorophore and the quencher and allowing the reporter dye to emit fluorescence. The fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

TaqMan probes were designed to increase the specificity of the reaction because detection and accurate quantification require high complementarity with the target sequence. The number of phytopathogenic fungi detected by this method has exponentially increased in recent years. Some examples are detailed in Table 2.

Modified probes have been designed to improve the TaqMan reaction specificity such as MGB probes that include a Minor Groove Binding group at the 3′ end raising the *Tm* of the hybrid. That allows the use of shorter and more specific probes. The high specificity of MGB probes make them very suitable for specific detection of fungal species based on SNPs (Massart et al., 2005). For the same purpose, primers or probes can be synthesized with a lock nucleic acid (LNA), which are modified nucleotides that form methylene bridges after binding to the target DNA (Braasch & Corey, 2001). That provokes the lock of the duplex structure, improving their binding affinity and stability and allowing the use of higher annealing temperatures. LNA primers have been used for the specific detection of *P. ramorum* (Tomlinson et al., 2007) and the multiplex detection of species of *Phytophthora* (Bilodeau et al., 2009). The high specificity of TaqMan probes may compromise however the accuracy of the detection and quantification due to the existence of interstrain variability in the target sequence that may result in failure to detect or underestimation of the amount of DNA targets in the sample.

In addition to its high specificity, one of the advantages of the TaqMan chemistry is that probes can be labeled with different reporter dyes (FAM, VIC, TET, TAMRA, HEX, JOE, ROX, Cy5, Texas Red, etc) which allows multiplexing detection of two or more distinct pathogens in the same reaction increasing throughput (Aroca et al., 2008; Bilodeau et al., 2009). However, for this porpose the synthesis of different probes is required making the multiplex detection analysis more expensive. The types and number of fluorescent labels that can be used depend upon the detection capabilities of the real-time instrument used. Additionally, designing of real-time multiplex assays may be difficult. Special attention must be focus on avoiding primer competition that could strongly drop the levels of specificity and sensitivity. In fact, TaqMan multiplex assays ussually show lower detection sensitivity than single reactions.

*b.2 Hairpin probes*

Molecular Beacons (MB) (Tyagi & Kramer, 1996) are specific oligonucleotide probes (15-40-mer) flanked by two complementary 5-7-mer arms sequences, with a fluorescent dye covalently attached to the 5′ end and a quencher dye at the 3' end. When the molecular beacon is in an unbound state the arms form a stem/loop structure in which the fluorophore

| Pathogen | Real-time chemistry | Reference | Host plant |
|---|---|---|---|
| *Biscogniauxia mediterranea* | TaqMan | Luchi et al., 2005 | Oak |
| *Botrytis squamosa* | TaqMan | Carisse et al., 2009 | Onion |
| *Chalara fraxinea* | TaqMan | Ioos et al., 2009 | Ash trees |
| *Colletotrichum acutatum* | TaqMan | Garrido et al., 2009 | Strawberry |
| *Colletotrichum gloesporioides* | TaqMan | Garrido et al., 2009 | Strawberry |
| *Colletotrichum* spp. | TaqMan | Garrido et al., 2009 | Strawberry |
| *Discula destructiva* | TaqMan | Zhang, N. et al., 2011 | Dogwood |
| *Fusarium avenaceum* | TaqMan MGB | Kulik et al., 2011 | Cereals |
| *Fusarium equiseti* | Molecular Beacons | Macía-Vicente et al., 2009 | Barley |
| *Fusarium foetens* | TaqMan | De Weerdt et al., 2006 | Begonia |
| *Fusarium graminearum* | TaqMan | Demeke et al., 2010 | Wheat<br>Barley |
| *Fusarium oxysporum* f. sp. *lycopersici* and its races | TaqMan MGB | Inami et al., 2010 | Tomato |
| *Fusarium poae* | TaqMan MGB | Kulik et al., 2011 | Cereals |
| *Fusarium tricinctum* | TaqMan MGB | Kulik et al., 2011 | Cereals |
| *Fuscoporia torulosa* | Scorpions | Campanile et al., 2008 | Oak |
| *Gremmeniella abietina* | TaqMan | Børja et al., 2006 | Spruce |
| *Guignardia citricarpa* | TaqMan | van Gent-Pelzer et al., 2007 | Citrus fruit |
| *Macrophomina phaseolina* | TaqMan MGB | Babu et al., 2011 | Chickpea<br>Soybean<br>Pigeon pea |
| *Mycosphaerella graminicola* | TaqMan MGB | Bearchell et al., 2005 | Wheat |
| *Phaeoacremonium aleophilum* | Taqman multiplex | Aroca et al., 2008 | Grapevine wood |
| *Phaeoacremonium mortoniae* | Taqman multiplex | Aroca et al., 2008 | Grapevine wood |
| *Phaeoacremonium parasiticum* | Taqman multiplex | Aroca et al., 2008 | Grapevine wood |
| *Phaeoacremonium viticola* | Taqman multiplex | Aroca et al., 2008 | Grapevine wood |
| *Phaeospora nodorum* | TaqMan MGB | Bearchell et al., 2005 | Wheat |
| *Phialophora gregata* | TaqMan | Malvick & Impullitti, 2007 | Soybean<br>Soil |
| *Phoma tracheiphila* | TaqMan | Demontis et al., 2008 | Citrus |
| *Phoma tracheiphila* | TaqMan | Licciardello et al., 2006 | Citrus |
| *Phomopsis* sp. | TaqMan | Børja et al., 2006 | Spruce |
| *Phytophthora citricola* | TaqMan multiplex | Schena et al., 2006 | Forest trees |
| *Phytophthora erythroseptica* | TaqMan | Nanayakkara et al., 2009 | Potato |
| *Phytophthora kernoviae* | TaqMan multiplex | Schena et al., 2006 | Forest trees |

| Pathogen | Real-time chemistry | Reference | Host plant |
|---|---|---|---|
| *Phytophthora pseudosyringae* | TaqMan multiplex | Tooley et al., 2006 | Rododendron and other host species |
| *Phytophthora quercina* | TaqMan multiplex | Schena et al., 2006 | Forest trees |
| *Phytophthora ramorum* | TaqMan multiplex | Bilodeau et al., 2009 | Oak |
| | TaqMan | Hughes et al., 2006 | *Parrotia persica* |
| | TaqMan multiplex | Schena et al., 2006 | Forest trees |
| | Scorpion and Molecular Beacon | Tomlinson et al., 2007 | |
| | TaqMan multiplex | Tooley et al., 2006 | Rododendron and other host species |
| *Phytophthora* spp. | TaqMan | Tomlinson et al., 2005 | Rhododendron |
| | TaqMan multiplex | Bilodeau et al., 2009 | Oak |
| *Plasmopara viticola* | TaqMan | Valsesia et al., 2005 | Grapevine |
| *Pochonia chlamydosporia* | Molecular Beacon | Macía-Vicente et al., 2009 | Barley |
| *Puccinia coronata* | TaqMan | Jackson et al., 2006 | Oat |
| *Puccinia graminis* | TaqMan | Barnes & Szabo, 2007 | Cereals |
| | | | Grasses |
| *Puccinia recondita* | TaqMan | Barnes & Szabo, 2007 | Cereals |
| | | | Grasses |
| *Puccinia striiformis* | TaqMan | Barnes & Szabo, 2007 | Cereal |
| | | | Grasses |
| *Puccinia triticina* | TaqMan | Barnes & Szabo, 2007 | Cereals |
| | | | Grasses |
| *Pyrenophora teres* | TaqMan MGB | Leisova et al., 2006 | Barley |
| *Pyrenophora teres* f. *maculata* | TaqMan MGB | Leisova et al., 2006 | Barley |
| *Pyrenophora teres* f. *teres* | TaqMan MGB | Leisova et al., 2006 | Barley |
| *Pythium vexans* | TaqMan | Tewoldemedhin et al., 2011 | Apple |
| *Rhynchosporium secalis* | TaqMan, LNA | Fountaine et al., 2007 | Barley |
| *Rosellinia necatrix* | Scorpion | Ruano-Rosa et al., 2007 | Avocado |
| *Thielaviopsis basicola* | TaqMan | Huang & Kang, 2010 | Tobacco |
| | | | Soil |
| *Ustilaginoidea virens* | TaqMan | Ashizawa et al., 2010 | Rice |
| | | | Soil |

Table 2. Examples of real-time PCR based on specific fluorescent labelled probes or primers for detection of plant pathogenic fungi (last 6 years).

and the quencher are in close proximity and fluorescence is quenched. When the probe hibridises to the target sequence the complementary arms separate, thus allowing the emission of fluorescence and hence making possible the detection and quantification of the target sequence. Because the stem/loop structure is very thermostable molecular beacons must have a high specificity to hybridise to a target. This makes the chemistry appropriate for the detection of single nucleotide differences in mutation and SNP analyses. Molecular beacons have allowed real-time specific quantification of *Fusarium equiseti* and *Pochonia chlamydosporia* (a nematode parasitic fungus) in barley roots (Macía-Vicente et al., 2009).

Scorpions® are bifunctional molecules in which an upstream hairpin probe is covalently linked to a downstream primer sequence (Whitcombe et al., 1999). The hairpin probe contains a fluorophore at the 5′ end and a quencher at the 3′ end. The loop portion of the scorpion probe is complementary to the target sequence. During the amplification reaction the probe becomes attached to the target region synthesized in the first PCR cycle. Following the second cycle of denaturation and annealing, the probe and the target hybridise resulting in separation of the fluorophore from the quencher and an increase in the fluorescence emitted. Improvement of Scorpions sensitivity has been achieved by placing the quencher in a separate oligonucleotide (Scorpions bi-probes) allowing greater separation of fluorophore and quencher and giving stronger signals. As with all dye-probe based methods, Scorpion probes follow strict design considerations for secondary structure and primer sequence to ensure that a secondary reaction will not compete with the correct probing event. Scorpion technology has been used for the detection of *Rosellinia necatrix* in roots of different plant host species and soils (Ruano-Rosa et al., 2007; Schena & Ippolito, 2003), and for the detection of *Fuscoporia torulosa* in holm oaks (Campanile et al., 2008).

*c. Specific fluorescent labelled primers*

Unlike other real-time chemistries, in which the incorporation of the fluorescent dye increases with the increasing number of copies of the DNA target, Plexor system measures the decrease of the fluorescence over time by the quenching of the dye. One of the two primers is labelled with a fluorescent dye and modified with methylisocytosine (isodC) residue at the 5′ end, whereas the other primer is not. The dabcyl-iso dGTP (iso-dG) present in the real-time PCR reaction cocktail is incorporated at the position complementary to the iso-dC label acting as a quencher and reducing the fluorescence over time. A quantitative real-time using Plexor primers has been developed for the detection and quantification of *Verticillium dahliae* in potato (Atallah et al., 2007).

### 2.2 Isothermal amplification methods

An efficient and cost-effective alternative to PCR is the possibility of isothermal amplification that does not require thermocycler aparatus. Loop-Mediated Isothermal Amplification (LAMP) (Notomi et al., 2000) uses a set of six oligonucleotide primers with eight binding sites hybridizing specifically to different regions of a target gene, and a thermophilic DNA polymerase from *Geobacillus stearothermophilus* for DNA amplification. This technique can specifically amplify the DNA target using only a heated block in less than 1 hour. Amplification products can be detected directly by visual inspection in vials using SYBR Green I, or by measuring the increased turbidity (due to the production of large

amounts of magnesium pyrophosphate), as well as by electrophoresis on agarose gel. LAMP method is very suitable for field testing and potentially valuable to laboratories without PCR facilities. This isothermal method has been applied for the rapid detection of *Fusarium graminearum* in contaminated wheat seeds (Abd-Elsalam et al., 2011) and for the detection of *Phytophthora ramorum* and *P. kernoviae* in field samples (Tomlinson et al., 2007, 2010).

### 2.3 Fingerprinting

Fingerprinting approaches allow the screening of random regions of the fungal genome for identifying species-specific sequences when conserved genes have not enough variation to successfully identify species (McCartney et al., 2003). Fingerprinting analyses are generally used to study the phylogenetic structure of fungal populations. However, these techniques have been also useful for identifying specific sequences used for the detection of fungi at very low taxonomic level, and even for differentiate strains of the same species with different host range, virulence, compatibility group or mating type.

#### 2.3.1 Restriction fragment length polymorphism (RFLP)

RFLP involves restriction enzyme digestion of the pathogen DNA, followed by separation of the fragments by electrophoresis in agarose or polyacrilamide gels to detect differences in the size of DNA fragments. Polymorphisms in the restriction enzyme cleavage sites are used to distinguish fungal species. Although DNA restriction profile can be directly observed by staining the gels, Southern blot analysis is usually necessary. DNA must be transferred to adequate membranes and hybridised with an appropriate probe. However, the Southern blot technique is laborious, and requires large amounts of undegraded DNA. RFLPs have been largely used for the study of the diversity of micorrhizal and soil fungal communities (Thies, 2007; Kim Y. T. et al., 2010; Martínez-García et al., 2011). Although used for differentiation of pathogenic fungi (Hyakumachi et al., 2005) this early technique has been progressively supplanted by other fingerprint techniques based in PCR.

PCR-RFLP combines the amplification of a target region with the further digestion of the PCR products obtained. PCR primers specific to the genus *Phytophthota* were used to amplify and further digest the resulting amplicons yielding a specific restriction pattern of 27 different *Phytophthora* species (Drenth et al., 2006). PCR-RFLP analysis of the ITS region demonstrated the presence of different anastomosis group (AG) within isolates of *Rhizoctonia solani* (Pannecoucque & Höfte, 2009); It also allowed the differentiation of pathogenic and non pathogenic strains of *Pythium myriotolum* (Gómez-Alpizar et al., 2011). In other cases, the analysis of the ITS region by this technique failed in differentiating closely related species (e.g., clade 1c species such as *Phytophthora infestans* and *P. mirabilis*) (Grünwald et al., 2011).

#### 2.3.2 Random Amplified Polymorphic DNA (RAPD)

RAPD analyses rely on PCR amplification of the pathogen genome with short arbitrary sequences (usually decamers) that are used as primers. These primers are probably able to find distinct complementary sequences in the genome producing specific banding patterns. The resulting PCR fragments are then separated by electrophoresis to obtain fingerprints

that may distinguish fungal species varieties or strains (Welsh & McClelland, 1990; Williams et al., 1990). Some of the specific DNA fragments detected in a profile may be cut out of the gel and sequenced to obtain a SCAR (Sequence-characterized amplified region), into which specific primers can be designed for a more precise PCR detection. SCAR primers have been used for instance to specifically identify *Phytophthora cactorum* (Causin et al., 2005), *Fusarium subglutinans* (Zaccaro et al., 2007) and *Guignardia citricarpa* (Stringari et al., 2009) in infected plant material; to distinguish among several *formae speciales* of *Fusarium oxysporum* (Lievens et al., 2008); to differentiate the bioherbicidal strain of *Sclerotinia minor* from like organisms (Pan et al., 2010); and to establish two different groups in *Gaeumannomyces graminis* var. *tritici* (Daval et al., 2010).

RAPD results are also useful for the analysis of the genetic diversity among populations. Fingerprints are scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrices. Data are analyzed to obtain statistic coefficients among the isolates that are then clustered to generate dendrograms. RAPDs have been used to analyze the genetic diversity among different species and races of *Fusarium* spp. (Lievens et al., 2007; Arici & Koc 2010) and different pathotypes of *Elsinoë* spp. (Hyun et al., 2009). This technique has also been applied to differentiate fungi isolates according to their host plant (Midorikawa et al., 2008), enzyme production profiles (Saldanha et al., 2007) or geographical origin and chemotypes (Zheng et al., 2009).

The RAPD technique is rapid, inexpensive and does not require any prior knowledge of the DNA sequence of the target organism. Results obtained from RAPD profiles are easy to interpret because they are based on amplification or non amplification of specific DNA sequences. In addition, RAPD analyses can be carried out on large numbers of isolates without the need for abundant quantities of high-quality DNA (Nayaka et al., 2011). Disadvantages of this technique include poor reproducibility between laboratories, and the inability to differentiate non-homologous co-migrating bands. In addition, RAPDs are dominant markers so, they cannot measure the genetic diversity affected by the number of alleles at a locus, nor differentiate homozygotes and heterozygotes individuals. This is not an issue with haploid fungi, but it can be a problem with many basidiomycetes and oomycetes that are heterokaryons, diploids or polyploids (Fourie et al., 2011).

### 2.3.3 Amplified fragment length polymorphism (AFLP)

AFLP analysis (Vos et al., 1995) consists in the use of restriction enzymes to digest total genomic DNA followed by ligation of restriction half-site specific adaptors to all restriction fragments. Then, a selective amplification of these restriction fragments is performed with PCR primers that have in their 3′ end the corresponding adaptor sequence and selective bases. The band pattern of the amplified fragments is visualized on denaturing polyacrilamide gels. The AFLP technology has the capability to amplify between 50 and 100 fragments at one time and to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As with other fingerprinting techniques, no prior sequence information is needed for amplification (Meudt & Clarke 2007). The disadvantages of AFLPs are that they require high molecular weight DNA, more technical expertise than RAPDs (ligations, restriction enzyme digestions, and polyacrylamide gels), and that AFLP analyses suffer the same analytical limitations of RAPDs (McDonald et al., 1997).

Depending on the primers used and on the reaction conditions, random amplification of fungal genomes produces genetic polymorphisms specific at the genus, species or strain levels (Liu et al., 2009). As a result, AFLP has been used to differentiate fungal isolates at several taxonomic levels e.g. to distinguish *Cladosporium fulvurn* from *Pyrenopeziuz brassicae* species (Majer et al., 1996), *Aspergillus carbonarius* from *A. ochraceus* (Schmidt et al., 2004), and *Colletotrichum gossypii* from *C. gossypii* var. *cephalosporioides* (Silva et al., 2005); also to differentiate *Monilinia laxa* that infect apple trees from isolates infecting other host plants (Gril et al., 2008); and to separate non-pathogenic strains of *Fusarium oxysporum* from those of *F. commune* (Stewart et al., 2006). AFLP markers have also been used to construct genetic linkage maps e.g. of *Phytophthora infestans* (Van der Lee et al., 1997). Specific AFLP bands may also be used for SCAR markers development used in PCR-based diagnostic tests. Using SCAR markers Cipriani et al. (2009) could distinguish isolates of *Fusarium oxysporum* that specifically infect the weed *Orobanche ramose*. AFLP profiles have also been widely used for the phylogenetic analysis of *Fusarium oxysporum* complex (Baayen et al., 2000; Fourie et al., 2011; Groenewald et al., 2006).

#### 2.3.4 Microsatellites

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are motifs of one to six nucleotides repeated several times in all eukaryotic genomes (generally in non-coding regions). These nucleotide units can differ in repeat number among individuals and their distribution in the genome is almost random. Using primers flanking such variable regions PCR products of different lengths can be obtained. So, the microsatellites are highly versatile genetic markers that have been widely exploited for DNA fingerprinting. The advantages of SSRs are that they are multiallelic, codominant, highly polymorphic and several thousand potentially polymorphic markers are available. Moreover, it is possible the analysis of samples with limited DNA amounts or degraded DNA with high reproducibility. The microsatellites have a high mutation rate and are able to gain and lose repeat units by DNA-replication slippage, a mutation mechanism that is specific to tandemly repeated sequences (Schlöetterer, 2000). This characteristic can create difficulties for populations-genetic analyses. Other drawbacks of the SSRs include the requirement of a prior knowledge of the DNA sequences of the flanking regions and their cost and low throughput because of difficulties for automation and data management. Moreover, a high number of microsatellite loci are necessary for a reliable phylogenetic reconstruction. However, the next-generation sequencing technologies and multiplexing microsatellites solve, in part, these problems.

Microsatellites have been used for the study of the genetic diversity of plant pathogenic fungi within species e.g. *Ascochyta rabiei* (Bayraktar et al., 2007), *Ceratocystis fimbriata* (Rizatto et al., 2010), *Macrophomina phaseolina* (Jana et al., 2005), *Puccinia graminis* and *P. triticina* (Szabo, 2007; Szabo & Kolmer, 2007), *Sclerotinia subarctica* and *S. sclerotiorum* (Winton et al., 2007); and for genetic map construction, e.g. Zheng et al. (2008) constructed a genetic map of *Magnaporthe grisea* consisting of 176 SSR markers. In other experiment, microsatellite markers specific for *Phytophthora ramorum* were employed to distinguish between A1 and A2 mating types isolates of this pathogen from two different geographic origins (Prospero et al., 2004).

To reduce the cost of developing microsatellites a novel technique has emerged based on sequence tagged microsatellites (STMs). Each STM is amplified by PCR using a single

primer specific to the conserved DNA sequence flanking the microsatellite repeat in combination with a universal primer that anchors to the 5´-ends of the microsatellites (Hayden et al., 2002). STMs have been developed for the plant pathogens *Rhynchosporium secalis* (Keipfer et al., 2006) and *Pyrenophora teres* (Keipfer et al., 2007).

### 2.4 DNA hybridisation technology

The use of Southern blot or dot blot hybridisation techniques using selected probes from DNA libraries was a strategy for the identification of plant pathogens prior to the introduction of PCR-based methods with greater sensitivity, simplicity and speed (Takamatsu et al., 1998; Levesque et al., 1998; Xu et al., 1999). Nevertheless, new and revolutionary methods based in hybridisation have been recently developed for detection and differentiation of phytopathogenic fungi:

#### 2.4.1 DNA arrays

A DNA array is a collection of species-specific oligonucleotides or cDNAs (known as probes) immobilized on a solid support that is subjected to hybridisation with a labelled target DNA. Macroarrays are membrane-based arrays containing spotted samples of 300 µm in diameter or more. Microarrays uses higher density chips such as glass or silicon, or microscopic beads in where thousands of sample spots (less than 200 µm in diameter) are immobilised via robotisation. The target DNA is a labelled PCR fragment, amplified with universal primers, spanning a genomic region that includes species-specific sequences. Probe-target hybridisation is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets and the relative abundance of nucleic acids sequences in the target can be determined. DNA micro- and macro-arrays are generally used for gene expression profiling but are also powerful tools for identification and differentiation of plant pathogens (Anderson N. et al., 2006; Lievens & Thomma, 2005). Currently, it is one of the most suitable techniques to detect and quantify multiple pathogens present in a sample (plant, soil, or water) in a single assay (Lievens et al., 2005a). The specificity of the DNA array technology allows an accurate SNP detection. This characteristic is crucial for diagnostic application since closely related pathogens may differ in only a single base pair polymorphism for a target gene. Specificity of the assay depends on the number and position of the mismatch(es) in the oligonucleotide probes, the oligonucleotide sequence and the lenght of the amplicon target. For instance, mismatches at the 3′ end of the oligonucleotide must be avoided and center mismatches are usually the most discriminatory sites (Lievens et al., 2006). Furthermore, using longer amplicons as targets increases the sensitivity but decreases the specificity of the array hybridisation. For improving specificity and robustness, the use of multiple oligonucleotides for a single pathogen and the use of multiple diagnostic regions are desirable. One of the main drawbacks of this technique is however, the lack of sensitivity. To reach sensitive detections, PCR amplification before array hybridisation is required, biasing the results through the species that are more represented in the sample.

This technology has been applied for detecting oomycete plant pathogens by using specific oligonucleotides designed on the ITS region (Anderson N. et al., 2006; Izzo & Mazzola, 2009). Another ITS and rRNA genes-based microarrays allowed the multiple detection and quantification of tomato pathogens (*Verticillium*, *Fusarium*, *Pythium* and *Rhizoctonia*)

confirmed by real-time PCR analysis (Lievens et al., 2005b), the monitoring of *Phytophthora* species diversity in soil and water samples (Chimento et al., 2005), and the identification and differentiation of toxin producing and non-producing *Fusarium* species in cereal grains (Nicolaisen et al., 2005). Using a *cox* I high density oligonucleotide microarray Chen et al. (2009) could identify *Penicillium* species. Moreover, Lievens et al. (2007) could detect and differentiate *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *radicis-cucumerinum* pathogens by a DNA array containing genus-, species- and *forma specialis*-specific oligonucleotides.

Additionally, A DNA array for simultaneous detection of over 40 different plant pathogenic soilborne fungi and 10 bacteria that frequently occur in greenhouse crops has been developed. This array, called DNA Multiscan® (http://www.dnamultiscan.com/), is routinely used worldwide by companies that offer disease diagnostic services and advice to commercial growers.

### 2.5 Sequencing

As discussed above, morphological characteristics are not always enough to identify a pathogen. One of the most direct approaches to do that consists in the PCR amplification of a target gene with universal primers, followed by sequencing and comparison with the available publicly databases. In addition, new fungal species have been described by using sequencing approaches. However, the use of sequence databases to identify organisms based on DNA similarity may have some pitfalls including erroneous and incomplete sequences, sequences associated with misidentified organisms, the inability to easily change or update data, and problems associated with defining species boundaries, all of them leading to erroneous interpretation of search results. An effort for generating and archiving high quality data by the researchers community should be the remedy of this drawback (Kang et al., 2010). Other limitation of sequencing as diagnostic tool is the need to sequence more than one locus for the robustness of the result, and the impractical of this method in cases when rapid results are needed such as for the control or eradication of serious plant disease outbreaks. Nevertheless, the increase of sequencing capacity and the decrease of costs have allowed the accumulation of a high numbers of fungal sequences in publicly accessible sequence databases, and sequences of selected genes have been widely used for the identification of specific pathogens and the development of sequence-based diagnostic methods.

#### 2.5.1 Massive sequencing techniques

The Sanger sequencing method has been partially supplanted by several “next-generation” sequencing technologies able to produce a high number of short sequences from multiple organisms in short time. Massive sequencing technologies offer dramatic increases in cost-effective sequence throughput, having a tremendous impact on genomic research. They have been used for standard sequencing applications, such as genome sequencing and resequencing, and quantification of sequence variation. The next-generation technologies commercially available today include the 454 GS20 pyrosequencing-based instruments (Roche Applied Science), the Solexa 1G analyzer (Illumina, Inc.), and the SOLiD instrument (Applied Biosystems).

Pyrosequencing is a DNA sequencing technology based on the sequencing-by-synthesis principle. The technique is built on a four enzyme real-time monitoring of DNA synthesis by

chemiluminescence and a fifth protein, SSB, which can be included to enhance the quality of the obtained sequences and thereby prolong the read length. The detection system is based on the pyrophosphate released when a nucleotide is introduced in the DNA strand. Thereby, the signal can be quantitatively connected to the number of bases added (Ahmadian et al., 2006). The pyrosequencing principle is used by the 454 platform, the first next-generation sequencing technology released to the market by Roche Applied Science. 454 technology is based in emulsion PCR (Tawfik et al., 1998), which uses fixing adapter-ligated-DNA fragments to streptavidin beads in water-in-oil emulsion droplets. In each droplet the DNA fixed to these beads is then amplified by PCR producing about $10^7$ copies of a unique DNA template per bead. Each DNA-bound bead is placed into a ~29 µm well on a PicoTiterPlate, a fiber optic chip, and analyzed using a pyrosequencing reaction. The use of the picotiter plate allows hundreds of thousands of pyrosequencing reactions to be carried out in parallel, massively increasing the sequencing throughput. 454 platform is capable of generating 80–120 Mb of sequence in 200 to 300 bp reads in a 4 h run. This technology enables a rapid and accurate quantification of sequence variation, including mutation detection, SNP genotyping, estimation of allele frequency and gene copy number, allelic imbalance and methylation status. Pyrosequencing can be applied to any DNA source, including degraded or low-quality DNA. Disadvantages are short read lengths, which may be problematic for sequence assembly particularly in areas associated with sequence repeats, the need for expensive biotinylated primers, and the inability to accurately detect variants within long (~ 5 or 6 bp) homopolymer stretches. In addition, multiplexing, while possible, is difficult to design.

The pyrosequencing technology has not been widely applied for the control of fungal plant diseases yet. However, Nunes et al. (2011) applied 454 sequencing technology to elucidate and characterize the small RNA transcriptome (15 - 40 nt) of mycelia and appressoria of *Magnaporthe oryzae*. Thus, they propose that a better understanding of key small RNA players in *M. oryzae* pathogenesis-related processes may illuminate alternative strategies to engineer plants capable of modifying the *M. oryzae* small transcriptome, and suppress disease development in an effective manner. Another application of this new sequencing technology is the rapid generation of genomic information to identify putative single-nucleotide polymorphisms (SNPs) to be used for population genetic, evolutionary, and phylogeographic studies on non-model organisms. Thus, Broders et al. (2011) described the sequencing, assembly and discovery of SNPs from the plant fungal pathogen *Ophiognomonia clavigignenti-juglandacearum*, for which virtually no sequence information was previously available. Moreover, Malausa et al. (2011) described a high-throughput method for isolating microsatellite markers based on coupling multiplex microsatellite enrichment and 454 pyrosequencing in different organisms, such as *Phytophthora alni* subsp. *uniformis*.

The principle of the Illumina/Solexa system is also based on sequencing-by-synthesis chemistry, with novel reversible terminator nucleotides for the four bases each labeled with a different fluorescent dye, and a special DNA polymerase enzyme able to incorporate them (Ansorge, 2010). In the ABI SOLiD (Sequencing by Oligo Ligation and Detection), the sequence extension reaction is not carried out by polymerases but rather by ligases. In the sequencing-by-ligation process, a sequencing primer is hybridized to single-stranded copies of the library molecules to be sequenced. (Kircher & Kelso, 2010). These two above mentioned systems have not been currently used in studies on plant pathogenic fungi.

### 2.5.2 DNA barcoding

DNA barcoding is a taxonomic method that uses a short genetic marker in the DNA to identify an organism as belonging to a particular species. It has facilitated the description of numerous new species and the characterisation of species complexes. Current fungal species identification platforms are available: *Fusarium*-ID was created as a simple, web-accessible BLAST server that consisted of sequences of the *TEF 1* α gene from representative species of *Fusarium* (Geiser et al., 2004). Sequences of multiple marker loci from almost all known *Fusarium* species have been progressively included for supporting strain identification and phylogenetic analyses. Two additional platforms have been constructed: the Fusarium Comparative Genomics Platform (FCGP), which keeps five genomes from four species, supports genome browsing and analysis, and shows computed characteristics of multiple gene families and functional groups; and the Fusarium Community Platform (FCP), an online research and education forum. All together, these platforms form the Cyber infrastructure for *Fusarium* (CiF; http://www.fusariumdb.org/) (Park, B. et al., 2011).

For *Phytophthora* identification two web-based databases have been created: (i) *Phytophthora* Database (http://www.phytophthoradb.org/) based in nine loci sequences including the ITS region and the 5′ portion of the large subunit of rRNA genes; nuclear genes encoding 60S ribosomal protein L10, β-tubulin, enolase, heat shock protein 90, TigA fusion protein, and TEF 1α; the mitochondrial gene *cox* II and spacer region between *cox* I and *cox* II genes (Park, J. et al., 2008); and (ii) Phytophthora-ID (http://phytophthora-id.org/) based on sequences of the ITS and the *cox* I and *cox* II spacer regions (Grunwald et al., 2011). Additional web-based databases are available including UNITE (http://unite.ut.ee/index.php), an ITS database supporting the identification of ectomycorrhizal fungi (Koljalg et al., 2005); TrichOKey (http://www.isth.info/tools/molkey/index.php), a database supporting the identification of *Hypocrea* and *Trichoderma* species (Druzhinina et al., 2005); and BOLD (http://www.boldsystems.org/) containing ITS and *cox* I databases from oomycetes (Ratnasingham et al., 2007; Robideau et al., 2011). Consortium for the Barcode of Life (CBOL) is an international collaborative effort which aims to use DNA barcoding to generate a unique genetic barcode for every species of life on earth. The *cox* I mitochondrial gene is emerging as the standard barcode region for eukariotes.

## 3. Fungicide resistance

Despite extensive fungicide use in the previous 90 years, resistance emerged as a practical problem as recently as 1970. The incidence of resistance has been restricted largely to systemic fungicides that operate to biochemical targets (single-site inhibitors). These included several of major groups of fungicides: sterol demethylation, bezimidazoles, pyrimidines, phenylamines, dicarboximides, carboxanilides and morpholines.

The resistance to toxic compounds is a genetic adaptation of the fungus to one or more fungicides that leads to a reduction in sensitivity to these compounds. This phenomenon, described as genotypic or acquired resistance, is found in numerous fungi that have been sensitive to the fungicide prior to exposure. Fungicide resistance can be acquired by single mutations in genes of the pathogen or by increasing the frequency of subpopulations that are naturally less sensitive. We have to differentiate between resistance and natural or intrinsic insensitivity of species that are not sensitive to the action of these compounds (Delp & Bekker,

1985; Brent, 1995). The term "lower sensitivity" is used in practical situations where there is a decreased sensitivity to a fungicide without an effect on field performance. The term "field resistance" is used when both the level of resistance and frequency of resistant strains are high and coincident, resulting in noticeable decline of field performance (Hewit, 1998).

It is to be expected that the evolutionary and dynamic progress of selection should eventually produce fungi that are resistant to fungicides. Therefore fungicide resistance is variable, with fungi developing resistance to some fungicides more rapidly than to others and in some cases no resistance had been reported after long periods of fungicide use.

In fungicide resistance, we should consider how pathogens have the ability to evolve resistant and how fungicides varies their susceptibility to resistance. Hence, resistance risk is determined by the target pathogen and selected fungicide.

Populations of fungi are so diverse that mechanisms of resistance may be present within the population before the fungicide is applied. The rate of resistance development in a population depends on the resultant mechanism of resistance such as how resistance characters are inherited, the epidemiology of the fungus, the environment, and the persistence of selective pressure.

### 3.1. Mechanisms of fungicide resistance

Fungicide resistance can be conferred by various mechanisms including: (I) an altered target site, which reduces the binding of the fungicide; (II) the synthesis of an alternative enzyme capable of substituting the target enzyme; (III) the overproduction of the fungicide target; (IV) an active efflux or reduced uptake of the fungicide; and (V) a metabolic breakdown of the fungicide. In addition, some unrecognized mechanisms could also be responsible for fungicide resistance.

#### 3.1.1 The reduction of intracellular concentration of antifungal compound

Currently, the most widespread hypothesis to explain the reduced levels of toxic products in the cell is based on active efflux of these compounds by ABC transporters (ATP binding cassette) and MFS (Major Facilitator Superfamily) (Hayashi et al., 2001, 2002a, 2002b, 2003; Stergiopoulos et al., 2002a, 2002b; Stergiopoulos & de Waard, 2002, Vermeulen et al., 2001). ABC and MFS transporters are the most studied so far and have been described in many fungi such as *Aspergillus nidulans, Botrytis cinerea, Mycosphaerella graminicola, Magnaporthe grisea, Penicillium digitatum*, etc (Andrade et al., 2000a, 2000b; del Sorbo et al., 2000; Nakaune, 2001; Schoonbeek et al., 2003; Stergiopoulos et al., 2002a; Stergiopoulos & de Waard, 2002; Vermeulen et al., 2001; Yoder and Turgeon 2001; Zwiers et al., 2003). Its function is to prevent or reduce the accumulation of compounds and therefore to avoid or minimize their toxic action (Bauer et al., 1999, Pao et al., 1998). ABC transporters comprise a large family of proteins and are located outside the plasma membrane or within the cell in intracellular compartments as vacuoles, endoplasmic reticulum, peroxisomes and mitochondria. They can carry a wide variety of toxic against the gradient (Del Sorbo et al., 2000; Theodoulou, 2000). ABC transporters include systems both capture and removal, generally showing activity on a wide range of substrates (fungicides, drugs, alkaloids, lipids, peptides, sterols, flavonoids, sugars, etc), but there are specific transporters for substrates (Bauer et al., 1999, Del Sorbo et al., 2000).

The overexpression of ABC and MFS genes plays an essential role in the resistance of chemically unrelated phenomenon described as multidrug resistance or MDR drugs (Del Sorbo et al., 2000; White, 1997). This phenomenon has been observed in a wide variety of organisms and can be a real threat to the effective control of fungal pathogens (Fling et al., 1991). In phytopathogenic fungi, these transporters can also be a virulence factor in providing protection against defense compounds produced by the plant or mediating the secretion of host-specific toxins. Also play a major role in determining the baseline sensitivity to fungicides and other antifungal agents. (De Waard, 1997; Stergiopoulos et al., 2003a, 2003b).

In *P. digitatum*, causal agent of citrus green rotten, four ABC transporters have been identified so far. ABC transporters PMR1 (Hamamoto et al., 2001b; Nakaune et al., 1998) and PMR5 (Nakaune, 2001; Nakaune et al., 2002) have been studied previously. Disruption of PMR1 in sensitive and resistant strains results in an increased sensitivity to DMIs and other compounds (Nakaune et al., 1998; Nakaune et al., 2002). However, the introduction of resistant strains from PMR1 restores the resistance while the introduction of PMR1 from sensitive strains does not have the same effect and does not restore the resistance. This suggests that although PMR1 plays an important role in the sensitivity of *P. digitatum* against DMIs alone does not explain the differences between sensitive and resistant strains (Hamamoto et al., 2001). Another of the genes studied is PMR5. This gene has highly homologous to PMR1, and also to *atrB* from *Aspergillus nidulans* and *BcAtrB* from *Botrytis cinerea* (Schoonbeek et al., 2003), however, is strongly induced by benzimidazoles, resveratrol and other compounds, but not for DMIs. This shows the different substrate specificity of both proteins and may play an important role in providing protection against natural or synthetic toxic compounds (Nakaune et al., 2002).

Sequence analysis in all four ABC transporter genes in several sensitive and resistant strains revealed no mutations in PMR1, PMR3 and PMR4, and point mutations only were observed in both the promoter and coding regions of PMR5 in multiple resistant strains (TBZ- and DMI-resistant) (Sánchez-Torres & Tuset, 2011). But no explanation was ascertained for the absence of sequence changes relating to fungicide resistance in the other ABC transporters, particularly in the PMR1 gene given that transcription of PMR1 has proven to be strongly activated in the presence of different fungicides (Hamamoto et al., 2001b; Nakaune, 2001; Nakaune et al., 1998).

To date, MFS transporters have been described in several fungi, e.g. *Botrytis cinerea* shows a broad spectrum of resistance to different fungicides and their expression has been induced by many of them, particularly noteworthy *Bcmfs4* induction in the presence of strobilurin (trifloxiestrobin) (Hayashi et al., 2002a, 2002b, 2003; Schoonbeek et al., 2003; Vermeulen et al., 2001). All this means that these transporters are potential candidates for the study of factors involved in resistance based on active eflux of these toxic compounds since they have remarkably broad substrate specificity although they can also transport specific compounds.

Recently, five different MFS transporters have been identified and characterized in the postharvest phytopathogenic fungus *Penicillium digitatum* (PdMFS1-PdMFS5). Sequence analysis of these five genes revealed different genomic structure and although all genes seem to be implicated in pathogenicity, only 2 out of five MFS transporters confirmed to be involved in fungicide resistance (Sánchez-Torres et al., submitted). Therefore, the most recent thought for fungicide resistance based on active efflux of these toxic compounds is

now discussed. These results suggest that many genes could be involved in the mechanisms conferring fungicide resistance to phytopathogenic fungi and some are fungicide-dependent.

From a practical standpoint, the fact that ABC and MFS transporters determine the baseline sensitivity to fungicides, are responsible for MDR and can act as virulence factors implies that these carriers are an attractive target for chemical control. In this context, inhibitors of these transporters could improve the effectiveness of control and reduce the virulence of fungal pathogens.

#### 3.1.2 Changes in binding target that causes a reduced affinity of the compound fungicide

The most extent mechanism to confer DMI resistance involved mutations of *CYP51* gene, the target enzyme of DMIs fungicides and has been described for a large number of pathogens such as *Botrytis cinerea* (Albertini et al., 2002, Albertini & Leroux, 2004), a substitution of Phe for Tyr at position 136 (Y136F) was found in *Uncinula necator* (Délye et al., 1997) and also in *Erisiphe graminis* f. sp. *hordei* (Délye et al., 1998). Two single nucleotide mutations of *CYP51* resulting in amino acid substitutions Y136F and K147Q in *Blumeria graminis* were also found (Wyand & Brown, 2005). Different mutations were also found in *Tapesia* sp (Albertini et al., 2003), *Penicillium italicum* (Joseph-Horne & Hollomon, 1997), *Ustilago maydis* (Butters et al., 2000) and *Blumeriella jaapii* (Ma et al., 2006)

Similarly, mutations have been described in the cytochrome b gene that lead to change of its corresponding protein G143A, conferring resistance to strobilurin (Avila-Adame & Koller, 2003a, 2003b; Gisi et al., 2000, Zheng et al., 2000, Zhang, Z. et al., 2009) or the amino acid substitution in the β-tubulin target protein involved in development of resistance to benzimidazoles in *Botrytis cinerea* (Banno, 2008), *Venturia inaequalis* and *Penicillium italicum* (Koenraadt et al., 1992), *Monilinia fructicola* (Ma et al., 2003a), *M. laxa* (Ma & Michailides, 2005), *P. expansum* (Baraldi et al., 2003) and *P. digitatum* (Sánchez-Torres & Tuset, 2011).

#### 3.1.3 Over-expression of the target of union of fungicide

P45014DM increased by over-expression of the *CYP51* gene has been described as a mechanism of resistance to azoles. In *Penicillium digitatum* a unique 126-bp sequence in the promoter region of *CYP51* was tandem repeated five times in resistant isolates and was present only one in sensitive isolates. This provided a quick and easy method to detect DMI-resistant strains of *P. digitatum* (Hamamoto et al., 2001). Insertions in the promoter were also found in *Blumeriella jaapii* (Ma et al., 2006), *Venturia inaequalis* (Schnabel & Jones 2001) and in *Monilinia fructicola* (Luo et al., 2008) and recently another insertion of 199-bp was found in *P. digitatum CYP51A* gene (Ghosoph et al., 2007) and *PdCYP51B* gene (Sun et al., 2011; Sánchez-Torres et al., submitted) leading to resistant phenotypes.

#### 3.1.4 Compensation of the toxic effects of the fungicide by altered biosynthetic or metabolic pathway

This phenomenon has been described both in the case of DMIs because they exert their toxic effect by depletion of ergosterol and the accumulation of C14 methylated precursors and in the case of strobilurins since the presence of AOX (alternative oxidase) allows the use an

alternative route in mitochondrial respiration (Gisi et al., 2000; Schnabel et al., 2001; Wood & Hollomon, 2003).

### 3.2 Molecular detection of fungicide resistance in phytopathogenic fungi

The procedures for detecting fungicide resistance using conventional methods are labor-intensive and time-consuming if large numbers of isolates have to be tested. Advances in molecular biology have provided new opportunities for rapidly detecting fungicide resistant genotype once the mechanisms of resistance have been elucidated at a molecular level. Several molecular techniques, such as PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), allele specific PCR, allele-specific real-time PCR and massive sequencing techniques (all above described) have been used successfully to detect fungicide-resistant genotypes of several plant pathogens. Many examples of these techniques have been reported.

#### 3.2.1 PCR detection of DMI-resistant isolates

PCR has revolutionized molecular biology and diagnostics and has become a fast tool for detecting fungicide-resistant pathogens. In *Penicillium digitatum*, the DMI resistance resulted from over-expression of the *PdCYP51* genes driven by a tandem repeat of five copies of a 126 transcriptional enhancer (Hamamoto et al., 2000), or insertion of 199-bp in the promoter region of *PdCYP51A* (Gosoph et al., 2007) or by the presence of 199-bp enhancer in the promoter region of *PdCYP51B* (Sun et al., 2011). Based on the DNA sequence of the *PdCYP51*-genes, PCR primers have been developed which are able to distinguish not only between sensitive and resistant DMI strains but allow identification of molecular mechanism that takes place (Hamamoto et al., 2001; Gosoph et al., 2007; Sánchez-Torres & Tuset, 2011; Sun et al., 2011).

#### 3.2.2 PCR-RFLP and primer-introduced restriction analysis PCR (PIRA-PCR)

PCR amplification followed by restriction enzyme analysis (PCR-RFLP) is a general technique to detect a point mutation that alters a restriction enzyme site. This method has been used to rapidly detect benzimidazole-resistant isolates of *Monilinia laxa* from stone fruit and almond crops in California (Ma & Michailides, 2005) and can also detect azoxystrobin-resistant isolates of *Alternaria alternata, A. tenuissima*, and *A. arborescens* within a few hours (Ma et al., 2003b). Since the PCR-RFLP is an easy and rapid technique, these assays have been developed for detecting benzimidazole resistance in *Botrytis cinerea* (Luck & Gillings, 1995), *Cladobotryum dendroides* (McKay et al., 1998), *Helminthosporium solani* (Cunha & Rizzo, 2003; McKay & Cooke, 1997), and for detecting strobilurin resistance in *Blumeria graminis* f.sp. *hordei* (Baumler et al., 2003), *Erysiphe graminis* f. sp. *tritici* (Sierotzki et al., 2000), and *Podosphaera fusca* (Ishii et al., 2001).

Although PCR-RFLP is a simple method for detection of point mutations, some target DNA fragments may not contain a restriction endonuclease recognition sequence at the site of point mutation. Thus, primer-introduced restriction analysis PCR (PIRA-PCR) has become a useful method to create diagnostic artificial RFLPs (Haliassos et al., 1989). A PIRA-PCR assay was developed for detecting carpropamid resistant *Magnaporthe grisea* (Kaku et al., 2003).

### 3.2.3 Allele-specific PCR and quantitative allele-specific real-time PCR

Allele-specific PCR is another simple and rapid method for detecting point mutations. Usually, one of two PCR primers used in an allele specific amplification is designed to amplify preferentially one allele by matching the desired allele and mismatching the other allele at the 3′ end of primer.

Allele-specific PCR assays have been developed for detecting benzimidazole-low resistant isolates of *Monilinia laxa* (Ma & Michailides, 2005), and azoxystrobin-resistant isolates of *Alternaria alternata, A. tenuissima*, and *A. arborascens* (Ma & Michailides, 2004). Additionally, allele-specific PCR assays have been developed for the rapid detection of strobilurin-resistant isolates of *Blumeria graminis* f. sp. *tritici* (Fraaije et al., 2002) and *Mycosphaerella fijiensis* (Gisi et al., 2002), and DMI-resistant isolates of *Erysiphe graminis* f. sp. *hordei* (Dèlye et al., 1997).

Real-time PCR technique can be used to quantitatively determine the amount of target DNA in a sample. An allele-specific real-time PCR assay has been used to follow the dynamics of *QoI* resistant allele *A143* in field populations of *Blumeria graminis* f. sp. *tritici* before and after fungicide application (Fraaije et al., 2002). A real-time PCR assay to rapidly detect azoxystrobin-resistant *Alternaria* has been developed in California pistachio orchards (Ma & Michailides, 2005). Additionally, a real-time PCR using the *Alternaria* specific PCR primer pair can quantify both resistant and sensitive alleles in the same tested samples, thereby enabling a rapid determination of frequencies of the azoxystrobin-resistant allele in *Alternaria* populations.

## 4. Conclusion

Advances in the development of molecular methods, especially PCR technology have provided diagnostic laboratories with powerful tools for detection and identification of phytopathogenic fungi. Molecular techniques have also contributed to elucidate the phenotypic and genetic structure within species and the complexity of plant and environment fungal populations. 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 fungal diseases. These advances have been complemented by the development of new nucleic acids extraction methods, increased automation, reliable internal controls, multiplexing assays, *online* information and *on site* molecular diagnostics. Nevertheless, molecular diagnostic tools should be complemented with other techniques, either traditional culture-based methods or the newly emerged proteomic, a promising tool for providing information about pathogenicity and virulence factors that will open up new possibilities for crop disease diagnosis and crop protection.

On the other hand, fungicides continue to play a key role in strategies for the control of diseases in crops, and the development of resistance in the target pathogens is a continuing risk. This fact leads to many losses as control systems are not longer effective. Therefore, the better understanding on mechanisms developed during fungicide resistance is essential for a better management of chemical control, environment and human health.

Great advances have been made in the development of molecular methods to identify and monitor resistance of plant pathogens to fungicides. The highly sensitive methods can

improve our ability of studying the evolution of fungicide resistance at the population level. Molecular techniques can be also developed based on the different fungicide mechanisms to rapidly detect resistant isolates. Furthermore, a timely detection of resistance levels in populations of phytopathogenic fungi in a field would help growers make proper decisions on resistance management programs to control plant diseases.

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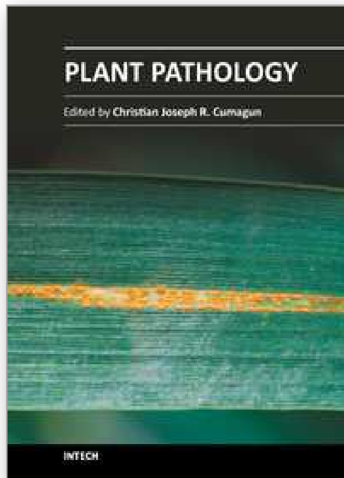


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