Plant Pathology in the 21st Century

Maria Lodovica Gullino
Peter J.M. Bonants *Editors*

# Detection and Diagnostics of Plant Pathogens

Springer

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# Foreword

This volume continues the series of books on "Plant Pathology in the 21st Century", which started in 2010, in cooperation with the International Society for Plant Pathology and contains the lectures given at the 10th International Congress of Plant Pathology (ICPP 2013) held in Beijing, August 25–30, 2013.

At such Congress, several sessions dealt with aspects of detection and diagnosis of plant pathogens, which represent two fundamental steps in disease management decisions.

For both detection and diagnosis, new tools and technologies have been developed, which are often replacing old methodologies, permitting to be faster, more specific and more precise.

A quick and reliable detection method in combination with decision support systems is fundamental in order to reduce the damages caused by old and new pathogens, thus permitting to reduce the number of treatments and to contain the potential losses.

Molecular methods are available for a number of pathogens and the volume provide good examples of application in different production sectors. Innovative techniques and methods will be described to detect and identify different targets: destructive and non-destructive, air- or soil-borne, human and plant pathogens, in plants or seed-born, native or emerging pathogens, on-site or lab-based. All to support international organizations to secure global trade and agriculture all over the world.

We believe that, besides representing a written testimony of ICPP 2013, this book will be useful for all plant pathologists as well as students in advanced courses.

We wish to thank all the colleagues who accepted to be part of this book, Zuzana Bernhart and her group at Springer for their continuous support and Laura Castellani for her skilful technical assistance.

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# Part I
# Technologies

# New Developments in Identification and Quantification of Airborne Inoculum

**Steph Heard and Jonathan S. West**

**Abstract** Airborne spores initiate many fungal diseases of crops but can occur with a patchy spatial distribution or with a variable seasonal timing. New diagnostic methods are available for use on spores sampled in air to give a rapid and on-site warning of inoculum presence or to monitor changes in genetic traits of pathogen populations, such as the race structure or frequency of fungicide-resistance. Increasingly, diagnostic methods used on-site or even integrated with air sampling equipment are being developed. These include fluorescence and image analysis methods, DNA-based methods such as qPCR, isothermal DNA amplification (LAMP and recombinase polymerase amplification), antibody-based methods (fluorescence microscopy and resonance imaging, ELISA, lateral flow devices, and biosensors such as holographic or SRi sensors) and biomarker-based methods (such as detection of volatile or particulate toxins or other metabolites by electrochemical biosensor). By allowing a rapid detection, these methods can offer a direct warning of the presence of inoculum to direct disease control decisions. Air samplers are often used within crops, just above the crop canopy, or on aircraft (including UAVs) or on tall buildings. Their location affects the threshold of spore concentrations that translates to disease risk. The optimal deployment of air samplers varies according to how widespread the pathogen is, the type of air sampler used (particularly the rate of airflow sampled for volumetric devices) and the importance or value of the crop.

**Keywords** Optical sensing • Remote sensing • Biosensor • Inoculum detection • Immunological detection • DNA-based detection • Biomarker

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## Introduction

Plant disease epidemics occur at different times and locations. Infection processes are directly influenced by the complexities of inoculum availability, growth stage of susceptible crop plants, and weather patterns (McCartney et al. 2006). Air-dispersed spores have been attributed to both long-distance introductions of pathogens over continental scales and local spread within or next to the field where the spores were produced (Brown and Hovmøller 2002; Gregory 1973). This can lead to small foci of infections when inoculum arrival is rare, uniform disease when inoculum is ubiquitous and disease gradients initiated from local areas of inoculum. Detection of airborne pathogen propagules that initiate disease is key information to drive decisions on crop protection measures and can also be used as an unbiased and easily obtained sample to monitor changes in genetic traits of pathogen populations.

Many airborne crop diseases are seasonal due to production of fruiting bodies only under certain conditions and spores are often released only after specific weather events. For example ascospores of many fungal plant pathogens are produced in fruiting bodies on crop debris in fields or field margins. These are often actively released after wetting by rain, which allows the spores to be ejected into more turbulent air to enhance dispersal and causes locally deposited spores to have perfect conditions for infection on wet leaves if the plant they have landed on is susceptible. In contrast, asexually-produced spores of rust fungi and powdery mildews are usually dry spores, passively released in windy conditions. This causes spore release to occur mainly during the day. Dispersal of airborne spores are often very local to the source but some spores are dispersed to altitudes over 10 km and have been documented to have dispersed over continental scales, often crossing oceans (Pady and Kapica 1955). Those that remain close to the ground may still be dispersed over 100 km, with spores within 100 m altitude often settling under gravity at night in relatively still air, falling at speeds between 0.03 cm $s^{-1}$ to over 2 cm $s^{-1}$ (Gregory 1973). Although spores of some species of fungi are very sensitive or not long-lived, others are able to tolerate UV light, desiccation and freezing (Gregory 1952). As a result, RT-PCR methods, which detects the more transient RNA in spores or culturing methods can be used to assess whether sampled spores are still viable (West et al. 2008). Even spores that are rain-splash dispersed may become aerosolised, particularly as a fine spray, caused by a combination of rain and strong winds. It has been reported that in Florida, the Asiatic citrus canker bacterium, *Xanthomonas axonopodis* pv. *citri* could be dispersed for up to 7 miles in severe rainstorms and tropical storms (Gottwald et al. 1992, 2001). Some fungal spores and bacteria are associated with ice-nucleation or enhanced condensation of water on their surfaces, leading to snow or rain formation, which returns the spores to ground from high altitudes (Fröhlich-Nowoisky et al. 2009; Morris et al. 2013). Recently various molecular methods suggest that bacteria are more prevalent in air than thought previously, when culturing methods were routinely used for assessment (<10 % of bacteria are able to be cultured in general media). For example, Morris et al. (2013) presented a

highly plausible explanation that ice nucleating *Pseudomonas syringae* affected the hydrological cycle, by inducing rainfall, by ice nucleation activity to aid their dispersal, with isolates taken from a wide range of habitats (upland lakes, snow, soil, rivers, rock and plant leaves) each having a high likelihood of possessing effector genes necessary for plant infection. Plant pathogen populations often comprise many cultivar-specific races that are subject to selection according to the extent of cultivation of particular crop varieties. Van der Wouw et al. (2010) demonstrated how air sampling integrated with qPCR can be used for monitoring changes in race structure of *Leptosphaeria maculans*. Similarly, mutations that confer resistance to fungicides can be monitored for research and applied purposes (Fraaije et al. 2005).

Types of air sampler used in plant pathology are reviewed in Jackson and Bayliss (2011) and West et al. (2008). These can include passive samplers, which collect a deposit of spores and other particles onto an adhesive surface (such as a vaseline-coated microscope slide) and more usually, volumetric samplers, which include impactors such as the Hirst and rotating-arm trap, or cyclone traps, filters, impingers (which are necessary for efficient collection of very small particles) and virtual impactors (Cox and Wathes 1995; Lacey and West 2006). Additionally a type of electrostatic spore trap, the ionic spore trap has been produced (http://ionicsporetrap.com/). These have different pros and cons regarding air flow rate sampled, power consumption, sample period, collection efficiency, period they can be left unattended and ease of processing the samples. Impactors were originally intended for microscopy or culturing methods but some newer air samplers (miniature cyclone, MicroTitre Immuno Spore Trap, and the Ionic spore trap) have been, designed specifically for analysis using non-visual methods such as immunological and molecular diagnostics and most if not all older types are adaptable for these downstream diagnostic assays. Increasingly, samplers that sample into tubes or other vials are used to make processing steps user-friendly and even to facilitate automated testing of samples. Lab-based diagnostics applied to air samples suffer the disadvantage of the delay taken to transport samples to the lab but in some cases, this remains the only method available and may be more cost effective if multiple target organisms can be detected. However, a key factor to enhance this approach further, is the rapidly developing area of using new diagnostics, such as biosensors, lateral flow devices and isothemal DNA assays, that can detect pathogens rapidly and on site. For more generic studies of pathogens and the air-spora community, particularly for unknown fungi, bacteria and viruses or viroids in other particles, lab-based methods such as terminal restriction fragment length polymorphism (TRFLP), denaturing gradient gel electrophoresis (DGGE) and next generation sequencing are often used.

Direct airborne inoculum-based disease forecasting networks have been established for a few pathogens in a few countries and is of particular value if the disease incubation period is long e.g. in Poland for *Leptosphaeria maculans* (www.spec.edu.pl), which uses a network of Hirst spore traps and for vegetable brassica diseases in England (http://www.syngenta-crop.co.uk/brassica-alert/), which uses multivial cyclones. The latter collects a daily air sample at each site but these are

only tested manually, using an on-site immunological test, if the weather data collected at the same site is conducive for infection. Direct inoculum-based forecasts are best suited to situations where the frequency of a problem is sporadic or spatially patchy, since diseases occurring every year will become routinely treated by farmers and rare diseases will not justify sampling expense. In addition, such a system is only necessary if there is no accurate weather-based forecasting system, if there is the possibility of severe economic yield loss and if there is a relatively cheap, rapid and reliable diagnostic method available. Finally the spatial variability of the target spores in relation to disease occurrence should be researched to optimise sampler location.

Optimal deployment of air samplers to assist disease control decisions is likely to vary according to the pathogen and crop system. It is not usually possible to use most types of air samplers for biosecurity purposes to detect very rare influx of an exotic species from a distant source due to dilution in the atmosphere (Jackson and Baylis 2011). However, some high volume spore traps such as the Jet spore trap, which samples 600 L/min (Burkard Manufacturing Co., Rickmansworth, UK) and the ionic spore trap (around 600 L/min) may be of use. Generally, once a pathogen has established a local sporulating focus of disease, it is more likely that the inoculum can be detected. The optimal deployment of air samplers varies according to the volume of air sampled by the device used, how widespread or common the pathogen is and the importance of the crop. Spore concentrations decline with distance from the source, usually a negative exponential or power function but other functions have been described. Usually these are similar in shape close to the source and vary from site to site and daily, according to wind speed, turbulence and crop canopy density. Usually it is not known exactly where the spore source is, and therefore which part of the concentration decline curve the sample was taken at. A relatively high concentration of spores could be caused by a very large distant source or a small source of spores very close to the sampler. As a result, care should be taken to interpret thresholds of spore concentrations to trigger disease control operations and normally this cannot be based on results from a single air sampler. However, some buffering against sampling effects, caused by releases of spores close to the sampler, can be achieved by mounting air samplers well above the ground or even on the roof of a tall building. For common plant pathogens it is possible to infer presence of airborne inoculum over a regional scale from a single air sampler located at rooftop height.

As farms become larger and growers become more reliant on mechanised equipment, the need for automated and user friendly diagnosis tools will be greater. Grower-friendly methods of pathogen detection need to be practical, readily available and cost effective. Methods to detect airborne spores of plant pathogens are becoming increasingly feasible due to advances in DNA-based diagnostics, antibody-based diagnostics, biosensors, and wireless communications. Diagnostic methods include spectral, fluorescence and image analysis methods for detection and identification of spores in air (or water) at the microscopic scale, but also DNA-based methods (PCR, qPCR, isothermal DNA amplification, and next generation sequencing particularly for bacteria, viruses and viroids), antibody-based

methods (fluorescence microscopy and resonance imaging, ELISA, lateral flow devices, and biosensors such as holographic or SRi sensors) and biomarker-based methods (such as detection of volatile or particulate toxins or other metabolites by electrochemical biosensor). These are discussed below.

## Culturing and Microscopy

A classical method for identification of fungal and oomycete spores is to use diagnostic keys with microscopy. In many cases it is only possible to identify spores to a genus and additional methods such as inducing spore germination to observe the germ tube branching pattern may be needed by sampling onto an impaction surface coated with a thin film of media. Spores can also be taken from culture plates used with semi selective media in impactors that collect spores onto agar plates, such as the Andersen sampler (West et al. 2008) or the Burkard portable air sampler (http://www.burkard.co.uk/portsamp.htm). Typically, a sample is placed into a humid chamber with or without a light source such as near-UV light depending on the species to encourage sporulation. However, these methods can be very time consuming, and impossible for obligate pathogens. Various staining methods, including immuno-labelling can be used to aid identification of spores. Visual recognition systems are available for automatic identification and counting of microscopic particles, particularly plant spores and pollen (www.aeromedi.org/home) and are being developed for common fungal spores. However, it remains extremely difficult to identify many plant pathogens to the species level. Unless a bioassay is used (e.g. inoculation onto a differential set of host plants), it is not possible to determine qualities that may be needed for quarantine purposes such as mating type, biotype or virulence group, for which nucleic acid-based methods are needed (described below).

## Fluorescence and Particle Recognition Systems

In addition to methods used in combination with microscopy, systems used for automatic identification and counting of spores can be based on combinations of their optical properties such as size, shape, light scatter, pigmentation and UV fluorescence. These spore qualities can be assessed by passing airborne spores between a laser or LED source and appropriate sensors (Stanley et al. 2011) or spores can also be captured into liquid and processed into flow cytometry equipment for similar optical assessment (Day et al. 2002). Other devices that are mainly used by the military can detect microbes in samples (typically air) in near real-time. Most are not able to identify any specific organism but simply detect an elevated level of any viable microbes in the air. For example the Biotrace BBDS system (http://www.adpsa.co.za/Biotrace/Biotrace%20Intro.htm#Biotrace) uses microbial

ATP in the cells or spores to produce fluorescence from the luciferase enzyme derived from fireflies. Other systems can detect a single organism such as *Bacillus anthcis* (Anthrax spore sensor; http://www.nano.org.uk/news/733/). These approaches offer real time detection of biological particles but usually are not specific and at best will indicate only a type of spore rather than a species. They are better applied to clean-room, clinical and bioterrorism applications where real-time detection of a potential threat is necessary but could play a role in targeting use of more specific diagnostic methods.

## Nucleic Acid-Based Diagnostics

Typically an air sample may need cell lysis (e.g. by shanking with microscopic glass beads) and DNA extraction and purification to make DNA available for an assay, but some species of fungi and bacteria, particularly with delicate spores, can be detected directly by PCR (Williams et al. 2001). Various nucleic acid based assays have been developed, generally using a probe or primers that bind to a specific sequence of DNA or RNA, present in the target. This sequence will be specific to a taxon such as a kingdom, species, or a genetic trait present in a population. An amplification step may or may not be used to enhance the detection of the target sequence. The main methods involving amplification steps are polymerase chain reaction or PCR and quantitative- or real-time PCR, which have been used extensively since the late 1990s for identification and quantification of plant pathogens and the study of genetic traits such as fungicide resistance or toxin production. PCR requires the design and testing for specificity and sensitivity of primers, either by sequencing DNA of one or more isolates or searching sequence databases. PCR methods use Taq polymerase and a cocktail of nucleotides plus potassium and magnesium or manganese ions in a reaction mixture with the sample DNA. The process involves thermal cycling, typically heating to 90 °C to separate all double-stranded DNA, followed by rapid cooling to more moderate temperatures (usually 50–70 °C) to allow binding of primers (forward and reverse) to specific sections of DNA that complement their designed sequence, only if the target sequence is present. This is followed by subsequent extension of the bound primer mediated by Taq polymerase to make a double stranded product. Multiple cycles of the specific thermal regime, creates a mass of PCR product or amplicon, which can be visualised on a gel by electrophoresis. For qPCR, addition of a DNA-specific fluorescent dye such as SYBR green allows quantification of the amplicon by fluorescence measurement after each replication cycle and this is compared to the fluorescence of known amounts of the pure pathogen's DNA which are run at the same time as standards. A better qPCR method for improved specificity is the Taqman qPCR, which uses in addition to primers, an oligonucleotide probe which is labelled at the 5′ end with a fluorophore and has a 'quencher' molecule on the 3′ end that prevents fluorescence while the quencher is in close proximity to the fluorophore. The probe binds to the amplicon but is cleaved into

separate nucleotides by the polymerase that is extending from the 5′ to 3′ direction, which separates the fluorophore and quencher to allow fluorescence, which is measured after each cycle (e.g. Yang et al. 2004). Alternatively nucleic acids and particularly PCR products can be visualised by other fluorescent probes such as scorpions (Sharkey et al. 2004) and molecular beacons (Tyagi and Kramer 1996). These can be multiplexed so that different Taqman, scorpion or molecular beacon probes with different fluorescent labels can be used to quantify different nucleic acid targets in one test (Sharkey et al. 2004) allowing a single sample to be tested for the presence of several pathogens. With these techniques, because PCR can be inhibited by the presence of certain chemicals or the DNA extraction process may not have worked, care is needed to include controls (Peccia and Hernandez 2006; McDevitt et al. 2007). This can comprise testing a sub-sample of DNA with consensus fungal primers that will detect all fungi, which are to be expected in any outdoor air sample, or spiking a sub-sample with a known amount of the target DNA to test for inhibition.

The use of isothermal DNA amplification methods such as Loop-mediated isothermal amplification (LAMP; Notomi et al. 2000) and recombinase polymerase amplification (Piepenburg et al. 2006; www.TwistDX.com) is increasingly becoming a tool for pathogen detection. These methods use enzymes to separate double stranded DNA without the need for heating the sample to 90 ºC as with traditional PCR and therefore are more analogous to DNA replication in living cells. This allows the reaction to take place at a single temperature, typically around 65 °C for LAMP and 37 °C for the TwistDX method. This makes the method more suitable for lightweight, portable devices as heating small tubes to a set temperature is relatively easy compared to thermal cycling and has lower energy requirements. A hand-held device is currently under development (Li et al. 2013). Although the technique may not be quite as sensitive as qPCR, it is often possible to be used on relatively crude DNA extracts, which also facilitates on-site detection using portable equipment. A deposit of air particulates, for example, can be lysed chemically in an extraction buffer and then the liquid can be used in the reaction (Boonham et al. 2013; Li 2013; Mahaffee et al. 2013). Results are obtained typically in 5–10 min. PCR and LAMP can be used to detect RNA (for detection of viruses or active gene expression in eukaryotes) by using a reverse-transcriptase to double the single-stranded RNA into DNA (known as RT-PCR; Freeman et al. 1999).

## *DNA and Protein Arrays and Microarrays*

DNA microarrays consist of a solid surface, such as a glass slide, onto which is printed a known arrangement of tiny dots of nucleic acid primers. They are commonly known as DNA-chips or DNA-arrays. The dots of primers each bind to specific nucleic acids from an air or other environmental sample, or to different genes of a target species under investigation. As a result, DNA of an environmental sample containing multiple species and genotypes of particular species can be

tested in one assay. DNA multiscan is one example, which is able to detect multiple plant pathogenic bacteria, oomycetes and fungi (http://www.dnamultiscan.com/en/home.html). Similarly, species specific protein targets can also bind to arrays of different antibodies, bound to a solid surface in a similar way to that described for ELISA (below). The term 'lab on-a-chip' has been used for systems that miniaturise processes used in diagnostics such as DNA or protein arrays. These are microfluidic devices in which liquids are moved through tiny pathways carved into glass slides. It is claimed that this gives faster reactions due to tiny volumes used taking advantage of diffusion and large surface area to volume ratios and ability to heat sections of the chip using minimal power.

### *Genomics-Based Detection*

This approach is certainly not a rapid test but is of particular interest for identification of unknown bacteria, viruses and viroids, especially because no previous sequence data of the organism is needed, nor specific primers or probes and there is also no need to culture the organism (Rodoni et al. 2013), which is important since only around 10 % of bacteria are culturable (Pace 1997). Next generation sequencing (NGS) (various platforms exist such as Solexa, 454 Roche, Illumina and Ion Torrent) pyrosequencing and metagenomics have been used (Rodoni et al. 2013; Hopkins et al. 2013). Currently, sequencing to identify an unknown pathogen can cost as little as $850 and takes about 2 weeks (Olmos et al. 2013).

## Immunology-Based/Antibody-Based Detection Methods

Advances in rapid and cost effective antibody production over the last 10 years have allowed the development of antibody based detection systems for plant disease diagnosis. Antibody based diagnosis/detection systems have been designed to be used in the laboratory as well as in hand held devices to be used for on-site detection. These systems are based on the use of antibodies as high affinity ligands which will bind to species specific cell surface fragments or antigens or even whole cell substrates. Antibodies that can be used in various detection systems can be monoclonal or polyclonal. Monoclonal antibodies (mAb) will bind to a single site or epitope of the target fragment whereas polyclonal antibodies (pAb) will bind to multiple epitopes on a single antigen allowing more specificity for target detection. Routinely, mAbs and pAbs are produced by the injection of the whole cell/pathogen or surface fragment into a suitable animal. An increasingly popular antibody production method is the use of bacterial expressed recombinant fragments which include single chain variable fragments (scFv) (Skottrup et al. 2008). These fragments are a sixth of the size of standard antibodies (Lamberski et al. 2006) and maintain high specificity to the parental mAb. Using bacterial cultures for large