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Molecular Diagnostics in Plant Disease Diagnostic Clinics… What's the Status?

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Plant disease diagnoses are typically based on consideration of all the evidence available, such as environmental conditions, plant symptoms and pathogen signs, site or field history and cultivar or plant species affected. Often multiple diagnostic methods are used to confirm or support a suspected diagnosis, such as use of molecular diagnostic assays which determine the presence or absence of a disease causing agent. From an accurate diagnosis, steps can be taken to either treat or manage the disease including, plant or cultivar selection, change in cultural or agronomic practices that influence disease, pesticide applications, biological control agents and in some situations quarantine or destruction of plants and plant products. Molecular diagnostic assays not only play an important role in plant disease diagnostic clinics and inspection services, but also are an important part of many research programs related to disease etiology and epidemiology. In 1995, Putnam [1] reviewed diagnostic methods used in plant disease diagnostic clinics, concluding that "new applications are reported almost monthly and although some have been specifically applied to diagnostic situations, the methods are largely not suitable for use in general diagnostic laboratories". Over the last two decades DNA based resources and diagnostic tools have increased significantly, has this change in resources and technologies been reflected in the diagnostic assays used in plant diagnostic clinics?

Molecular diagnostics provide significant advantages and are often complimentary to traditional techniques of microscopy and culture based pathogen identification. Advantages of molecular diagnostics include ability to detect an organism without prior culturing, faster turn-around time, potential for high-throughput and ability to identify pathogen species or strains (i.e. detection of fungicide resistance alleles). Serology or immunoassay based techniques such as enzyme linked immunosorbent assay (ELISA) has formed the mainstay of the plant disease diagnosticians' molecular tool kit, particularly for the identification of plant viruses. Nucleic acid based techniques such as conventional PCR, nucleic acid hybridization, quantitative real-time PCR (qPCR) and isothermal loop mediated amplification (LAMP) [2], often provide improved specificity and sensitivity over immunoassays. However, in many instances there is still a large disconnect between development and implementation of nucleic acid based diagnostic assays. This disconnect exists for several reasons, including familiarity of the diagnostician with technologies of the assay, equipment requirement to the diagnostician, poorly validated assays and the fact that plant diagnostic laboratories typically process hundreds or thousands of samples a year often of very diverse hosts and pathogens [1]. The need to be familiar with a multitude of assays and to have primer and probe sets for the detection of each pathogen does not permit the diagnostician the ability to have all of the assays on hand. For these reasons molecular assays are typically only used for commonly encountered diseases or highly regulated pathogens. Another reason for the disconnect between published assays and their use in plant disease diagnostic clinics, is that diagnosticians are typically not thought of as the end user. Researchers developing diagnostic assays need to be more cognizant of assay end use.

Molecular assays within the diagnostic lab need to be rapid, accurate and robust and inexpensive to execute. PCR assays are typically inexpensive and robust, but require time post-PCR to electrophorese PCR product for assay interpretation, as such a PCR assay can take up to 7 hours from beginning to end. Real-time PCR (qPCR) assays have an advantage over traditional PCR in that a positive reaction can be determined in real-time without the need for post-analysis of PCR product, allowing results to be obtained within 2-3 hours. Loop mediated amplification (LAMP) [2], is a relatively new DNA amplification technique that has been little utilized in the diagnosis of plant diseases, but offers some advantages over PCR based methods. Due to the strand displacing nature of the polymerase used in LAMP, thermo-cycling is not required, as such LAMP assays can be run on simpler less expensive equipment such as a simple heat block or water bath. LAMP assays are also more rapid than qPCR assays and can be conducted in less than 1 hour and like qPCR can be quantitative [3].

Morphological identification in the plant diagnostic laboratory is typically only made to the genus level, due to time and difficulty in differentiating species within a genus with very close morphologies. Examples of organisms which are typically only identified to genus level within diagnostic laboratories are *Pythium*, *Phytophthora*, *Fusarium* and *Rhizoctonia*. These organisms are associated with seedling or root rot diseases, where disease symptoms do not usually suggest a particular species is involved. Routine identification of these pathogens to species level in the plant disease diagnostic clinic would facilitate not only an improved understanding of disease etiology and epidemiology, but also potentially improve management recommendations given the significant differences in pathogenicity, aggressiveness and fungicide sensitivity between species within a genus. Single strand confirmation polymorphism (SSCP) is a method that has been used to identify species within a genus. SSCP utilizes a common primer pair to amplify species within a genus such as *Phytophthora*. The amplified fragment is then denatured and separated on a polyacrylamide gel and the banding pattern is used to differentiate species [4]. Although the technique has proved valuable for research studies, it does have limitations in a diagnostic laboratory setting, such as time and effort to run and stain a polyacrylamide gel, the need for DNA from representative species for banding pattern comparison and the difficulty in databasing DNA patterns in polyacrylamide gels.

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DNA arrays offer great potential for the interrogation of a sample with hundreds or thousands of DNA probes. There are essentially two classes of DNA arrays, which are differentiated by the density of probes, into micro- or macro-arrays. Although both platforms have been investigated for the detection of pathogens, few of these are routinely utilized by diagnostic labs. Reasons for this include cost of the microarrays and availability of the macro-arrays, both arrays also require a lengthy hybridization step, a signal collection step and if detection of a pathogen from an environmental sample is required a PCR amplification step may be necessary prior to hybridization. A recent and exciting development for the simultaneous detection of multiple targets in a sample is the combination of an inexpensive array platform and simple low-cost CCD camera to capture amplification of LAMP assays in a microfluidic chip [5]. The concept for this device is for an inexpensive and disposable plastic microfluidic chip in which a sample can be interrogated with multiple LAMP assays (Syed Hashsham, *personal communication*).

The continued and dramatic decrease in sequencing costs opens possibilities for the development of molecular diagnostic assays. It may even be possible in the near future to sequence entire samples at a reasonable cost [6], utilizing bioinformatic tools to identify pathogen specific reads present in a sample. However, for this approach to be successful a curated database of reference sequences/genomes must be available. Currently GenBank (National Center for Biotechnology Information) does not allow 3rd party curation of deposited sequences, as such annotation errors are propagated at an alarming rate. This is also an issue for the frequently used ribosomal DNA internal spacer region (ITS) which is often sequenced by many laboratories for the identification of unknown fungal and oomycete cultures. However, there are efforts underway in the mycological community, for the development of curated databases for species identification such as Fusarium ID and Phytophthora ID, as well as larger more encompassing projects such as Assembling the Fungal Tree of Life (AFTOL) and Barcoding of Life Database (BOLD).

Although resources and techniques for molecular diagnostics have grown significantly over the last couple of decades, there is still a long way to go in the development and application of molecular diagnostics to assist the plant disease diagnostician. Ultimately, molecular diagnostic assays for use in plant disease diagnostic clinics need to be reliable, robust, inexpensive and easy enough to use that they compete with and complement traditional techniques. The challenge now resides with researchers to develop practical assays for use in a diagnostic setting.

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