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Conventional and advanced techniques for detection of seed borne pathogens

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Abstract

Food losses resulting from crop infections by pathogens such as bacteria, viruses, and fungi have long posed a significant challenge in global agriculture. Seeds are pivotal in the spread of plant pathogens to new areas and serve as carriers for their survival between growing seasons. Seed-borne diseases present a major biological hurdle in global seed production. Accurate and timely detection of soil pathogens is crucial for developing effective management strategies. Effective seed health testing methods are characterized by specificity, sensitivity, speed, simplicity, cost-effectiveness, and reliability. Traditional methods fall short in terms of sensitivity, specificity, and accuracy. Serological tests, including enzyme-linked immunosorbent assays (ELISA) and immunofluorescence microscopy, have been employed to detect seed-borne pathogens. However, these methods have limitations, such as the absence of species-specific antibodies and the potential for false positives due to the detection of nonviable pathogens. To overcome these challenges, nucleic acid-based detection methods have emerged as highly effective tools. Techniques such as BIO-PCR, immunomagnetic separation PCR (IMS-PCR), magnetic capture hybridization PCR (MCH-PCR), and rapid-cycle real-time PCR, along with advanced technologies like DNA chip technology, microsatellites, DNA barcoding, next-generation sequencing ((NGS), and nanotechnology, have significantly enhanced the reliability, specificity, sensitivity, and speed of pathogen detection in seeds. This article reviews both serological and modern detection techniques that have been reported to monitor and identify the seed-borne diseases which provide a comprehensive theoretical foundation for effective disease prevention and management.

Keywords: Seed-borne pathogens, crop infections, bacteria, viruses, fungi, seed health testing

Introduction

The global population is increasing rapidly, and feeding the ever-increasing population poses a serious challenge for agriculturalists around the world. The agriculture sectors could play a critical role in the 'Zero Hunger' mission by minimization of crop losses through development, right and timely adaption of priority based disease management system in sustainable agriculture. Seed is a basic and critical input in agriculture to ensure global food security. Roughly 90 percent of the crops grown all over the world are propagated by seed. However, seed can also harbour and spread pathogens, e.g. fungi, bacteria, nematodes, viruses etc., which cause devastating diseases. In many agricultural and horticultural crops, seed priming was used as the best tool to enhance germination rate and uniformity. Seed-borne pathogens represent a major threat to crop establishment and yield. Hence, timely detection and diagnosis is a prerequisite for their effective management. Effective seed health testing methods are characterized by specificity, sensitivity, speed, simplicity, cost-effectiveness, and reliability. While traditional techniques such as visual inspection, selective media, and seedling grow-out tests are useful for detecting certain pathogens, they often fall short in terms of sensitivity, specificity, and accuracy. Traditionally, the most prevalent techniques used to identify plant pathogens relied upon culture-based morphological approaches. These methods are laborious, time-consuming, and require wide knowledge of classical taxonomy. Other restrictions include the difficulty of some species to be cultured *in vitro*, and the incapability to accurate quantification of the pathogen (Ghosh *et al.*, 2015, Sharma *et al.*, 2015b) [12]. It is important fact that diagnose of large share of soil-borne diseases is often difficult, as symptomless host plants at early stages of infection is more

challenging. A large number of diseases are spreading or introducing into a new area via. propagative materials e.g. seed, rhizospheric soil of seedling, propagules etc. (Biswas *et al.*, 2009, Katan, 2017) ^[5, 16]. Hence, early and speedy detection of soil-borne pathogens is prerequisite for planting materials. Because it could be the part of a controlling measure through effectively linking this tool with a safeguard agricultural systems to prevent further spreading of the disease in an area where the disease was absent.

Early and reliable detection of such diseases is crucial in order to reduce the huge economic losses as well as valuable human lives. On the other hand, early disease detection in crops is very imperative, because practice of using chemicals or biocides as a curative measure after a severe infection with high incidence and severity causes enormous negative impact on environment and biological ecosystem (Padaria *et al.*, 2016, Sharma *et al.*, 2017) ^[27, 36].

Here, we review the most important tools for conventional and molecular detection of various soil-borne plant pathogens and its implementation in agriculture.

Features of best detection techniques

Many assays are available for detecting various seed-borne pathogens, but only a few meet the minimum criteria required for effective seed testing. According to Ball and Reeves (1991) ^[3], six key requirements should guide the selection of seed health testing methods:

1. **Speed:** While some accuracy may be sacrificed for quicker results, rapid tests should be followed by more definitive testing to ensure accuracy.
2. **Specificity:** The ability to accurately distinguish the target pathogen from other organisms that may be present on seeds from the field or storage, thereby avoiding false positives.
3. **Reliability:** Testing methods must be robust enough to ensure consistent and repeatable results across different samples of the same stock regardless of who performs

the test (within the bounds of statistical probability and sample variation).

4. **Simplicity:** The methodology should be streamlined to reduce potential errors and allow tests to be performed by staff who may not have advanced qualifications.
5. **Sensitivity:** The capability to detect target pathogens, even when they occur at low levels in seed stocks, but are still significant for field crops.
6. **Cost-Effectiveness:** The cost of testing should be within acceptable production margins for each crop.

Historically, depending on the common methods used to monitor the target pathogen, seed health tests have been divided into the four categories i.e. direct inspection, incubation tests, immunodiagnostic and molecular methods.

Conventional Methods

Direct Inspection Method

The direct inspection method is valuable for preliminary screening studies, particularly for avoiding heavily infected seed stocks. This method can reveal the presence or absence of pathogens but does not provide information about the viability of those pathogens. Direct examination of dry seeds offers a qualitative and semi-quantitative approach to assessing seed health. It typically involves detecting fungal fruiting structures under a stereomicroscope or observing the external effects of fungal pathogens on seeds.

This method is particularly effective for identifying sclerotia, galls, smut balls, discoloration, malformation, resting hyphae, fungal fruiting bodies (such as oospores, pycnidia, perithecia, and smut spores), and bacterial masses (Rao *et al.*, 2006) ^[31]. Seeds that are severely infected by certain organisms may exhibit reduced size or discoloration. For example, Egyptian hybrid rice seeds infected with *Bipolaris oryzae* may show light brown, black dots, dark brown, and brown spots (Ibrahim and El-Dahab, 2014) ^[13].

Table 1: Summary of seed signs or symptoms, associated fungi, and references for various crops affected by seed-borne pathogens

Sr. No.	Crop	Sign or Symptom on Seed	Fungi Associated	References
1	Carrot	Seed rot	<i>Alternaria radicina</i>	Gaur (2011) ^[11]
2	Chick pea	Reduction in seed size	<i>Ascochyta rabiei</i>	Gaur (2011) ^[11]
3	Chilli	Acervuli and microsclerotia	<i>Colletotrichum dematium</i>	Kumar <i>et al.</i> (2004) ^[19]
4	Cereals	Normal seed is replaced by sori of spores	Smut, bunt, or ergot in cereals	Warham <i>et al.</i> (1996) ^[41]
5	Soybean	Purple stain	<i>Cercospora kikuchii</i>	Khare (1996) ^[18]
6	Soybean	Fine cracks and mould, starting near the hilum	<i>Phomopsis longicolla</i>	Li (2011) ^[22]

Growing on test (Seedling symptom test)

Seeds are sown in greenhouse in suitable substrates (brickstone, sand or other sterile substrate, agar tubes, etc.) in different environmental conditions. This method is particularly useful when seedlings show symptoms in a short time.

Immunodiagnostic Methods

In 1918, serology was first used to identify bacteria. This technique is based on the principle that a mammal's immune system is stimulated to produce specific antibodies that detect and bind to antigens when foreign molecules, known as immunizing agents or antigens, enter the bloodstream (Schaad, 1979; Fox, 1993) ^[34, 8].

- **Antibody:** A protein produced by plasma cells in response to an antigen.

- **Antigen:** A substance that invades the body and triggers a process that can lead to disease.

Serology-based seed tests have several formats, including the commonly used enzyme-linked immunosorbent assay (ELISA) (McLaughlin and Chen, 1990) ^[25] and immunofluorescence microscopy (Franken, 1992) ^[10]. Currently, serology is the most widely used method for detecting seed-borne viruses, known for its sensitivity and robustness (Barba, 1986) ^[4].

ELISA

In ELISA tests, an antibody specific to a pathogen's protein (antigen) is added to a sample. The resulting reaction is indicated by a color change, signaling infection. For example, soybean mosaic virus and bean pod mottle virus

can be detected using ELISA (ISTA, 2007) ^[14]. While there are various forms of ELISA (Ball and Reeves, 1991) ^[3], the three main types are direct (double antibody sandwich DAS-ELISA), indirect, and competitive (Lange, 1986) ^[21]. The direct method is the most commonly used in seed health tests (Lange, 1986; Kazinczi and Horvath, 1998) ^[21, 17]. In this method, a seed (or seedling) extract (the antigen) is selectively trapped and immobilized by solid-phase-specific antibodies in microtiter wells. The enzyme-labeled antibody binds to the immobilized antigen, and after unbound enzyme-labeled antibody is washed away, the bound enzyme is detected by adding a suitable substrate (Clark, 1981) ^[6]. ELISA has successfully detected *Pantoea stewartii* in maize seeds (McCornack and Ragsdale, 2006) ^[24].

Immunofluorescence Microscopy

Immunofluorescence (IF) microscopy merges serological techniques with microscopic analysis. It is a highly recommended immunodiagnostic approach for detecting seed-borne pathogens, particularly bacteria. IF microscopy can be performed in two ways: indirect (Malin *et al.*, 1983) ^[23] and direct (Franken and Van Vuurde, 1990) ^[9]. In this method, immunofluorescence cell staining involves detecting an antigen under ultraviolet light after it has been stained with a homologous antibody conjugated to a fluorescent dye, such as fluorescein isothiocyanate. The level of fluorescence correlates with the amount of pathogen present in the seed preparation. For instance, *Pseudomonas syringae* pv. *phaseolicola* can be identified using direct immunofluorescence, while *Xanthomonas campestris* pv. *phaseoli* is detected using the indirect method.

Lateral Flow Devices

Perhaps, now days lateral flow devices (LFDs) is one of the most readily available farmer's friendly diagnostic tools. The advantages of these devices are that they are simple to use and results are quick, usually in less than 10 min. LFDs are most useful for diagnosis of plant viral diseases and those LFDs are commercially available. The LFDs are mainly based on the serological specificity of polyclonal or monoclonal antibodies to particular targeted pathogens. The sensitivity of LFDs varies with target and type of antibody used. An LFD-based test using an IgM monoclonal antibody to detect *Rhizoctonia solani* can detect as little as 3 ng ml⁻¹ of antigen, equal to the sensitivity of standard ELISA procedures (Thornton, 2008).

PCR-Based Detection Techniques

Molecular techniques Molecular techniques based on hybridization or amplification, and especially on Polymerase Chain Reaction (PCR), have been developed for the most important plant pathogenic viruses and bacteria. Despite having great sensitivity and specificity, molecular techniques had trouble being adopted for routine detection due to a lack of resilience (Van der Wolf *et al.*, 2001) ^[39].

PCR involves the enzymatic amplification of target DNA *in vitro*, and because of its specificity, speed, and sensitivity, it has been widely used in diagnosing numerous seed-borne pathogens. The invention of the polymerase chain reaction (PCR) revolutionized biological diagnostics, ushering in a new era for medical and veterinary pathogen detection, as well as introducing the potential for detecting pathogens in seeds. Since its inception, a variety of PCR-based detection methods have been developed and applied to seed-borne

pathogens. These methods, as discussed by Agarwal and Sinclair (1997) ^[1], include the polymerase chain reaction (PCR) itself, as well as other DNA-based detection systems such as real-time PCR, Immuno-magnetic Separation and PCR (IMS-PCR), Bio-PCR, Magnetic Capture Hybridization PCR (MCH-PCR), and DNA chip technology (microarrays). Below is a brief overview of these innovative detection methods.

Real-time PCR

Rapid-cycle real-time PCR, a recent development in PCR, promises to remove many of these obstacles and make PCR more useful for seed identification. In real-time PCR, a fluorescent signal is produced along with DNA amplification to provide an amplicon count-dependent fluorescence signal (Kurian *et al.*, 1999; Cockerill and Smith, 2002) ^[20, 7]. Real-time PCR seed detection assays have been reported for *A. avenae* subsp. *citrulli* in watermelon seeds and *Microdochium nivale* in wheat (*Triticum* spp.) seeds (Taylor *et al.*, 2002) ^[37].

IMS-PCR

In this method, small magnetic beads, coated with antibodies for a specific microorganism, are able to selectively bind target cells from suspensions containing a mixture of cells (Olsvik *et al.*, 1994; Walcott and Gitaitis 2000) ^[26, 40]. Captured cells can then be incubated on selective media in order to increase the amount of the target pathogen or, alternatively, they could be used directly for DNA extraction and PCR run. Though IMS-PCR is more efficient and sensitive than conventional PCR but it relies on specific antibodies which may not be always available. More-over, due to the difficulty of beads for capturing cells of filamentous fungi, it can be successfully used for bacteria only.

Bio-PCR

Bio-PCR involves cultivating target pathogens on selective media to preferentially amplify them over non-target microorganisms, followed by DNA extraction and PCR amplification (Schaad *et al.*, 1995) ^[33]. In this method, seed samples are either washed or crushed in a suitable buffer to extract seed-borne pathogens. Portions of the seed wash are then spread onto semi-selective media and incubated for 2 to 3 days. After incubation, colonies are collected, and DNA is extracted for PCR amplification using specific primers. For seed-borne fungi, seeds are incubated under high relative humidity to increase the mycelial mass of the target fungus before DNA extraction and PCR (Pryor and Gilbertson, 2001) ^[30].

The primary limitation of this technique is that it cannot be used for obligate pathogens, as they cannot be cultured on artificial media. Additionally, this method is time-consuming due to the necessity of well-defined selective media for pathogen growth. The Bio-PCR method can detect various plant pathogens, such as *Xanthomonas oryzae* pv. *oryzae*, *Pseudomonas syringae* pv. *phaseolicola*, and *Acidovorax avenae*.

Multiplexing

Multiplex PCR or RT-PCR is the amplification of multiple targets simultaneously in a single PCR by using several primer pairs specific for each target. Development of a

multiplex PCR or RT-PCR assay is often complex since primers must comply with several conditions: i) similar melting temperatures (similar length and G-C content) so that all primers can function under the same PCR conditions; ii) compatibility, avoiding cross-binding and competition; iii) similar sensitivity; and iv) flanking genomic regions of different sizes so that the amplicons of each target can be separated and visualized by gel electrophoresis. The last constriction can be avoided by using primers labelled with different color fluorescent dyes or coupling the PCR with hybridization with specific probes (James *et al.*, 2006) ^[15]. Multiplex PCR or RT-PCR have been used to identify the main viruses infecting a particular crop, such as tomato, tobacco, legumes, potato, ornamentals, cucumber and olive (Ali *et al.*, 2014; Pallás *et al.*, 2018)

Biochemical technique

FAME (Fatty Acid Methyl Esterase) Analysis

Fatty Acid Profiles (FAME Analysis) identification of bacterial pathogens of plants by fatty acid methyl ester analysis is usually performed on pure cultures of the organism. About 40 mg of wet cells is saponified and methylated. The fatty acid methyl esters (FAME) are extracted in an ether-hexane mixture and analyzed by gas chromatography. Areas of the resulting peaks on the chromatograms are calculated and compared with profiles of known reference strains by computer programs (Roy, 1988). For example, the organism responsible for an outbreak of bacterial spot of lettuce was defined as *Xanthomonas campestris* pv. *vitians* as the fatty acid profiles of the strains collected from the field matched this pathovar most closely (Pernezny *et al.*, 1995) ^[29]. In a more extensive study, Wells *et al.* (1994) ^[42] were able to differentiate the five species of *Erwinia* of the *amylovora* group as well as the four species of the *herbicola* group. Electrophoresis of soluble proteins from plant pathogens often gives rise to complex patterns, and these can be used for identification purposes. Instead of using a general protein stain, such as Coomassie Blue, a particular protein dye, which, for example, might have enzymatic activity, may be revealed by appropriate staining methods.

Conclusion

The conventional plant pathogen diagnostic techniques are simple to use with no need huge investment. The modern techniques need an expertise to carry out the detection process, also need huge investment. The current modern techniques demonstrate reproducible sensitivity and are generally much faster than conventional techniques. The Rapid and timely detection of the pathogens associated with the particular disease is critical for taking up of the proper management practices. Many advanced techniques like PCR based methods helpful to detect the pathogens even from asymptomatic plants. Some techniques suitable to use under field conditions like lateral flow assay. Some techniques detect very low concentration of pathogens. However, since no single method satisfies all or even most of the emerging criteria for faster, effective, reproducible and sensitive results, there is still an obvious knowledge gap in research in this field.

Conflict of interest

Not applicable

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