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Review on Seed Health Tests and Detection Methods of Seedborne Diseases

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Abstract
Seed is a small embryonic plant which is an efficient means of introducing plant pathogens into a new area as well as providing a means of their survival from one cropping season to another. Seed health is a well recognized factor in the modern agricultural science for desired plant population and good harvest. Seed-borne fungi are one of the most important biotic constrains in seed production worldwide. Seed health testing to detect seed-borne pathogens is an important step in the management of crop diseases. Seed health is a measure of freedom of seeds from pathogens. ISTA, ISHI and NSHS are three primary organizations that publish standardized seed health test methods. Specificity, sensitivity, speed, simplicity, cost effectiveness and reliability are main requirements for selection of seed health tests methods. PCR has many beneficial characteristics that make it highly applicable for detecting seedborne pathogens. Since seed serve as means of dispersal and survival of plant pathogens, it is critical to test its health before using it as planting material. Seed health testing and detection is a first line approach in managing seedborn diseases of plants.
Keywords: Embryonic plant, Modern agricultural, PCR**,** Plant pathogens, Plant population, Seedborne fungi

1. INTRODUCTION

Seed is a small embryonic plant enclosed in a covering called seed coat. It is the product of the ripened ovule of gymnosperm and angiosperm plants which occurs after fertilization and some growth within the mother plant (Wikipidia, Undated) . Seed is the basic unit of production for the world's food crop. In recent years seed has become an international commodity used to exchange germplasm around the world. Seed is, however, also an efficient means of introducing plant pathogens into a new area as well as providing a means of their survival from one cropping season to another (Walcott *et al*., 2006). Seed health testing is thus routinely carried out in most countries for domestic seed certification, quality assessment and plant quarantine (FAO, 2010). Seed health testing is an integral for all seed companies in disease risk management (ISF, 2010).

Seed health is a well recognized factor in the modern agricultural science for desired plant population and good harvest (Rahman *et al*., 2008). Seedborne pathogens are a continuing problem and may even be responsible for the re-emergence of diseases of the past as well as the introduction of diseases into new areas (Gitaitis and Walcott, 2007). Seedborne pathogens present a serious threat to seedling establishment (Walcott, 2003). In today's global economy, seed accounts more than ever for the movement of plant pathogens across vast distances, natural barriers, and political borders (Gitaitis and Walcott, 2007). The quality of planted seeds has a critical influence on the ability of crops to become established and to realize their full potential of yield and value (McGee, 1995).

Seed-borne fungi are one of the most important biotic constrains in seed production worldwide. They are responsible for both pre and post-emergence death of grains, affect seedling vigor, and thus cause some reduction in germination and also variation in plant morphology (Van Du *et al*., 2001; Rajput *et a*l., 2005; Niaz and Dawar, 2009). The seedborne pathogens may result in loss in germination, discolouralion and shrivelling, development of plant diseases, distribution of pathogen to new areas, introduction of new strains or physiologic races of the pathogen along with new germplasm from other countries and toxin production in infected seed (Agarwal and Gaur, Undated). Fungi outnumber all other types of pathogens that attack plants and cause a very serious economic impact on agricultural production due to their ability to induce diseases of cultivated crops that result in important yield losses (Paplomatas, 2006) .

There are three primary organizations that publish standardized seed health test methods for use in international trade. These are International Seed Testing Association (ISTA), International Seed Health Initiative (ISHI), and in the United States, the National Seed Health System (NSHS) (Munkvold, 2009). Two of the most important concepts in seed health testing are sensitivity and selectivity, which are inextricably linked. For example, increasing the selectivity of semiselective media may decrease the recovery efficiency of all or some strains of the target organism. In contrast, increasing selectivity may reduce the number of nontarget organisms that act as competitors and/or inhibitors that interfere with the assay, and thus increase the detection sensitivity (Roumagnace *et al*., 2000; Toussaint *et al*., 2001; Wydra *et al*., 2004). A semiselective medium may have a higher mean plating efficiency than a standard growth medium because standard media are complex and often become toxic, perhaps due to the accumulation of peroxides or other secondary metabolites (Block *et al*., 1998; Pataky *et al*., 1995).

Infection rate of seeds depending on some environmental conditions such as high relative humidity, suitable temperature and also high level of moisture content in seed is variable. The study of seed-borne pathogens is necessary to determine seed health and to improve germination potential of seed which finally leads to increase of the crop production. Seed health testing to detect seed-borne pathogens is an important step in the management of crop diseases (Hajihasani *et al*., 2012). In relation with those importance of seed health tests and detection of seedborne pathogens: the present review has the following objectives: to review the basic requirements of health testing and detection methods of seedborne pathogens.

2. SEED HEALTH TESTING AND DETECTION

2.1. Seed Health Testing

Seed health is a measure of freedom of seeds from pathogens. The presence or absence of seed-borne pathogens can be confirmed through the use of seed health testing (Agrawal, 1995). The term "seed health" includes the incidence in the seed lot of fungi, bacteria, viruses, and animal pests such as nematodes and insects. The test used depends on the organism being tested for and the purpose of the test quality assurance or phytosanitary purposes when seed is exported (ISTA, 2009b). It includes visual examination of seeds externally or internally, macro or microscopically for the presence of pathogens as well as incubating seeds on agar or moist blotter papers and identifying the pathogens microscopically (Warham *et al*., 1990). Many detection assays exist for different seedborne pathogens, however, few satisfy the minimum requirements for adequate seed tests. Ideally, seed assays should be sensitive, specific, rapid, robust, inexpensive and simple to implement and interpret (Walcott, 2003).

Seed testing is necessary for a number of reasons: to determine the quality of the seed based on a number of seed quality attributes; to provide a basis for price and consumer discrimination among seed lots and seed sources; to determine the source of a seed problem, thereby facilitating any corrective measure(s) that may be required; and to fulfil legal and regulatory requirements for certified seed classes and allow for seed movement across international boundaries (FAO, 2010). There are six main requirements for selection of seed health tests methods (Amare, 2007/8). These are:

- Specificity: the ability to distinguish the target pathogen from all organisms likely to occur on seeds from field or store, i.e. to avoid false positives.
- Sensitivity: the ability to detect target organisms, which are potentially significant in field crops at a low incidence in seed stocks.
- Speed: in some cases, small concession to accuracy may be necessary to ensure rapid results, but such results should be followed by more definite testing.
- Simplicity: the methodology should minimize the number of stages to reduce room for error and to enable tests to be performed by not necessarily highly qualified staff.
- Cost effectiveness: test costs should form part of acceptable production margins for each crop.
- Reliability**:** test methods must be sufficiently robust so that results are repeatable within and between samples of the same stock regardless of who performs the test (within the bounds of statistical probability and sample variation).

Historically seed health tests have been classified into the following four distinct groups based on the general techniques used to observe the target pathogen. Such as Direct Inspection, Incubation Tests, Examination of the embryo (embryo count method), Immunoassays and Molecular Methods.

2.1.1. Direct examination (inspection)

Direct examination or inspection of dry seed is a qualitative and semi-quantitative seed health testing method where either the fruiting structures of fungi are detected under stereomicroscope or effects of fungal pathogens on the physical appearance of the seed are seen (Mathur and Jorgensen, 1998). By this method, it is possible to detect sclerotia, smut balls, fupgal spores and other fructifications such as pycnidia, perithecia, etc (Rao and Bramel, 2000). If seeds are severely infected by some organisms they may be reduced in size or discoloured. For example maize seeds infected with Nigrospora have white streaks with black spore masses near the tips and sorghum seeds infected with acremonium wilt are completely deformed (Agarwal and Sinclair, 1997).

2.1.2. Incubation tests

The seeds are incubated for a certain period in the agar plate or blotter test under specific environmental conditions in order to allow pathogens on the seed to grow. Different fungi are identified by features such as the form, length and arrangement of conidiophores, size, septation and chain formation of conidia (Warham *et a*l., 1990).

2.1.3. Blotter tests/Seedling symptom tests

In seed health testing for seed-borne fungal pathogens the blotter test is no doubt one of the most important methods available (Limonard, 1966). Blotter tests are similar to germination tests in that seeds are placed on moistened layers of blotter paper and incubated under conditions that promote fungal growth. The seed may then be allowed to germinate and fungal seed-borne infections may manifest themselves by any pertinent signs or

symptoms. The manifestations of the pathogen are influenced by the environmental conditions during incubation. The blotter test gives an indication of the infection of the seed, as shown by the presence of mycelium and fruiting bodies, and, in some tests, infection of the germinated seedlings as demonstrated by symptoms on the young plants. In some tests seeds are incubated during which they are allowed to germinate and symptoms are observed (e.g. dark spots on the cotyledons of bean seeds infected by the anthracnose pathogen). In other tests the germination of seeds is deliberately suppressed to allow seed-borne infection to develop (e.g. to allow the pycnidia of seedborne *Phoma lingam* to develop on *Brassica* seeds, the herbicide 2,4 –D is applied before incubation, allowing greater numbers of seeds to be tested) (Limonard, 1966).

2.1.4. Agar plate

Agar plate is the most common method used for identification of seedbome fungi (Rao and Bramel, 2000). Incubation methods allow the detection of viable fungus material even at the preliminary phase of development of the fungus. This is done generally by placing seeds onto sterile agar media (potato dextrose or malt agars are most commonly used) to encourage the growth of seedborne fungi. Agar plate methods may be employed to quantitatively determine the fungal load such as CFU/gm of seed (dilution plate methods) or to qualitatively determine the species composition (direct plate method). The most common way to estimate quantity of fungal tissue in kernels is the dilution plate method. There are two variations of this method: the pour plate method which is used more frequently, and the spread plate method which is preferred at low sample contamination. On the other hand, the direct plating method is one of the best methods to determine the composition of the grain fungi as to genera and species. In this method whole kernels are placed on the surface of the culture medium after having the kernels surface disinfected. The direct plating technique can be recommended as a very effective procedure for determining internal colonization of kernels by fungi and consequently is a very useful tool for evaluating the quality of bulk grain. There are considerable variations in the application of the agar test primarily with regard to preparation of samples, choice of media, and temperature and duration of incubation. Acidic agar media may be used to reduce bacterial contaminants (Trojanowska, 1991). Sometimes bacterial colonies develop on the agar and inhibit fungal growth making identification difficult. This can be overcome by adding an antibiotic such as streptomycin to the autoclaved agar medium after it cools to 50-55°C (Rao and Bramel, 2000).

2.1.5. Examination of the embryo (Embryo count method)

Examination of dry seeds with the naked eye and at magnifications of 10 to 30 times reveals a number of plant pathogens that occur mixed with the seeds as fungus bodies (for example, sclerotic) or have converted the seed into fungus structures (e.g., ergots). Sclerotic of the fungi Sclerotinia and Typhula may be mixed with seeds of clovers, crucifers, grasses, and other crop seeds. The fungus Claviceps Purpurea, the cause of ergot of grasses, often is mixed with seed as ergots (Andersen and Leach, 2010). Staining methods are used for seedborne pathogens which cannot be detected by direct inspection or incubation methods. The standard method used in seed health testing is that of staining of barley embryos for the presence of loose smut (*Ustilago segetum* var. *tritici*) mycelium.

2.1.6. Immunoassays

Immunoassays present a more sophisticated approach to testing, with Enzyme Linked Immunosorbent Assays (ELISA) and immunofluorescence being most common. In ELISA tests, an antibody to a specific protein (antigen) in the pathogen is added to a sample and the reaction between them reflected in a color change which indicates infection. For example, soybean mosaic virus, bean pod mottle and other viruses can be detected using ELISA (ISTA, 2009b).

2.1.7. Molecular methods

Molecular biology methods for agricultural diagnostics have become an area of increasing interest recently. A significant flaw in the majority of the assays published to date is that they stop short of being of practical application in a routine plant-health context (McEwan and Mulholland, Undated). There are DNA-based molecular techniques, the most common being the polymerase chain reaction (PCR), which selectively increases pathogen DNA. Electrophoresis is then used to separate the DNA into different sizes, followed by staining. The incidence of pathogen DNA can be identified by comparisons with known samples (ISTA, 2009b).

2.2. Seed Health Detection Methods

Detection deals with establishing the presence of a particular target organism within a sample, with special emphasis on symptomless individuals (Lopez *et al*., 2003). In general seed health detection can be classified in to two major assays, such as the conventional seed detection assays and polymerase chain reaction-based seed detection assays.

2.2.1. Conventional seed detection assays

Testing seeds for plant pathogens can be a difficult task. Unlike infected vegetative plant tissues, infested seeds can be asymptomatic, making visual detection impossible. Additionally, pathogen populations on seeds may be low, and infested seeds may be nonuniformly distributed within a lot. Many detection assays exist for different seedborne pathogens, however, few satisfy the minimum requirements for adequate seed tests. Ideally, seed

assays should be sensitive, specific, rapid, robust, inexpensive and simple to implement and interpret. Seed assays have been developed based on different technologies including visual examination; selective media; seedling grow-out tests and serological techniques (Walcott, 2003).

2.2.1.1.Bioassays

Perhaps the oldest seed health assay is the grow-out. This procedure is highly selective as it relies on the specificity of the host pathogen interaction. Its sensitivity is less assured, as inoculation thresholds may vary depending on the plant cultivar being tested, fluctuations in environmental conditions, fertility, and other factors. Additionally, the ability of plant inspectors to reliably detect low incidences of disease is a critical factor, as it is often necessary to visually recognize single lesions in thousands of plants. Nonetheless, grow-outs are widely used and accepted as definitive in determining the infection status of a seedlot. Because grow-outs rely on symptom expression, a positive result usually is irrefutable evidence that the bacterium was present, viable, and pathogenic (Gitaitis and Walcott, 2007).

Testing for Aac, the bacterial fruit blotch pathogen, provides a good example of a routinely used grow-out test. Optimum environmental conditions for disease development are needed to ensure that the assay is effective and reliable, as well as to prevent cross contamination from other inoculum sources. Other factors include frequent disinfesting of planting trays, greenhouse floors, walls, and benches; using a commercial greenhouse potting mixture or steam-sterilized soil; creating conditions that are optimal for seed germination and seedling emergence; and choosing an appropriate sample size of a minimum of 30,000 seeds or 10% seedlot. Throughout the duration of the test, a minimum relative humidity of 70% should be maintained continuously and should not be allowed to fall below 50% for more than 12 h. Temperatures during the grow-out should ideally range between 30^{o}C –32◦C, never falling below 25◦C or exceeding 38◦C for more than 12 h. To further verify that conditions are favorable for symptom expression, an Aac-infested seed sample should be included as a check in an isolated area of the greenhouse. To eliminate the risk of cross contamination from this inoculum sources, an Aac strain with an antibiotic resistance marker or a unique DNA fingerprint should be used to infest the control seedlot. Potential problems associated with splash dissemination of the bacterium during irrigation should be avoided by adequate spacing and the erection of plastic barriers 60–90 cm high between seedlots. Also necessary are good management strategies to limit the passive dissemination of pathogens by insects, mites, or other vectors. These precautions are necessary to prevent contamination between seedlots, but secondary spread within a seedlot is desirable because it results in the development of infection foci that improve the chances of visual detection (Gitaitis and Walcott, 2007). After germination, there should be daily inspections of cotyledons and true leaves until termination of the assay. Each seedling should be examined for symptoms in areas well illuminated with natural light. Natural water congestion is common with certain plants and should not be mistaken for water soaking associated with disease development. All contact with seedlings should be avoided until the final inspection, except for removal of symptomatic seedlings for isolation and testing. When it is necessary to handle seedlings, workers' hands should be sanitized or disposable gloves should be worn and changed between seedlots. A seedling grow-out assay can be terminated after 3 weeks with no visisble symptoms; however, if symptoms are observed, bacteria should be isolated and subsequent diagnostic tests (e.g., immuno-strips, PCR and/or pathogenicity) should be used to confirm the identity of the pathogen (Gitaitis and Walcott, 2007).

2.2.1.2. Serological methods (Immunoassays)

Serological seed assays rely on antibodies (polyclonal or monoclonal) generated against unique antigens on the surfaces of plant pathogens (Hampton *et al*., 1990). Antibodies bind strongly and specifically to their antigens and can subsequently be detected by the enzymatic digestion of substrates or fluorescent tags. Serological assays do not require pure isolations of the pathogen and, hence, are applicable to biotrophic and necrotrophic seedborne pathogens. (Walcott, 2003). In the past serology was the most widely used detection assay for seedborne viruses and it has proven to be sensitive and robust (Barba, 1986; Bossennec and Maury, 1978; Delecolle *et al*., 1985; Falk and Purcifull, 1983; Pasquini *et al*., 1998). Serology based seed tests have several formats including the widely applied enzyme linked immunosorbent assay (ELISA) (McLaughlin and Chen, 1990) and immunofluorescence microscopy (Franken, 1992). Serological methods used to detect and identify bacterial pathogens include agglutination tests, immunofluorescence microscopy (IF), immunofluorescence colony-staining (IFC), enzyme-linked immunosorbent assays (ELISA), Western blot, lateral flow devices (e.g., immunostrips), flow cytometry, and immunocapture techniques such as immunomagnetic separation (IMS) (Munkvold, 2009).

The difficulty in establishing a threshold of positive fluorescent cells that can lead to disease development in the crop discouraged use of IF in the black rot seed certification program in Georgia. Both researchers and regulatory personnel concluded that IF resulted in too many false positives, perhaps due to binding of the bacteria to nonviable cells or naked antigenic determinants (Munkvold, 2009). Techniques such as IFC were developed to overcome the problem of potential false positives (Glynn *et al*., 2008). IFC seed health assay incorporates seed extract with an equal volume of agar medium. The mixture is incubated, dried, and

exposed to target bacterium-specific antibodies conjugated with a fluorescent dye. Colonies stained with the antibody-dye conjugate can be visualized with fluorescent microscopy, and bacteria inside the colonies can be isolated with a glass capillary tube and transferred to a suitable growth medium. ELISA also has been used for seed health assays. In general, ELISA, like the IF, is suspected of producing too many false-positive results (Munkvold, 2009). ELISA successfully detected *Pantoea stewartii* ssp. *stewartii* in maize seeds (McCornack and Ragsdale, 2006). The double antibody sandwich (DAS)-ELISA with polyclonal and monoclonal antibodies was most appropriate for seed health testing. Later, the method was used to quantify populations of *P. stewartii* ssp. *stewartii* in individual seeds by constructing a response curve relating absorbance values with numbers of bacterial CFU recovered (ISTA, 2007).

Flow cytometery (Broders *et al*., 2007a), another promising serological technique for use in seed health assays, automatically sorts and analyzes bacterial cells tagged with dye-conjugated antibodies, while in suspension. Several parameters can thus be determined within a few minutes by measuring the degree of light scattering and fluorescence emitted by thousands of individual cells recovered from an infected seed sample. As a stream containing tagged bacterial cells passes through a flow cell, a laser beam illuminates the cells and excites the fluorescent tags attached to the antibodies. By using different fluorescent detectors, several parameters such as cell size, granularity, and cell roughness can be measured simultaneously. If used in conjunction with fluorescent probes that target key enzymes, membrane potential, or respiratory activities, flow cytometry can also determine the viability of target cells. Immunomagnetic separation (IMS) PCR is another technique that takes advantage of antibody-antigen specificity. IMS uses small magnetic polystyrene beads coated with antibodies to sequester target cells from heterogenous mixtures (Ojeda and Verdier, 2000). After immobilization with a magnet particle concentrator, the immunomagnetic beads are rinsed to remove inhibitory compounds and nontarget bacteria. Template DNA can be released from captured cells by boiling and used for PCR or, alternatively, captured cells can be plated onto a semiselective medium. In assaying water-melon seed, Walcott *et al*. (2003) showed that IMS-PCR improved the detection threshold of *A. avenae* subsp. *citrulli* 100-fold when compared to conventional PCR. IMS, essentially a concentration step to efficiently increase the amount of target DNA in the PCR sample, was also successfully used to detect *P. ananatis* in onion seeds. IMS-PCR can reduce false-negative reactions by improving the efficiency and reliability of extracting PCR quality pathogen DNA from seeds, and eliminating seed compounds that can inhibit PCR (Munkvold, 2009).

Table 1. General features of seed detection assays including the time required for completion, sensitivity, ease of application, specificity, and applicability for the detection of fungi, bacteria and viruses.

Assay Time of Specificity	**Time of required**	Sensitivity	Ease of application	Specificity
Visual examination	5–10 min	Low	Simple and inexpensive (requires experience)	Low
Semiselective media	2–14 d	Moderate	Simple and inexpensive	Low–moderate
Seedling grow-out assay	2–3 weeks	Low	Simple, inexpensive and robust	Low
Serology-based detection	2–4 h	Moderate–high	Simple, moderately expensive and robust	Moderate–high
Conventional DNA extraction and polymerase chain reaction (PCR)	5–6 h	High	Complicated; easy to interpret, expensive	Very high
BIO-PCR (selective target colony enrichment followed by PCR)	2-3 d	Very high	Complicated, expensive	Very high
IMS-PCR (immunomagnetic separation and PCR)	2-5 h	Very high	Complicated, expensive	Very high
MCH-PCR (magnetic capture hybridization and PCR)	2-5 h	Very high	Complicated, expensive	Very high
Real-time PCR	40-60min	Very high	Complicated, expensive	Very high
DNA microarrays	6 h	Very high	Complicated, expensive	Very high

Source: Walcott, 2003. Where d (day); h (hours)

2.2.2. Nucleic Acid–Based Detection Methods

Polymerase chain reaction (PCR) is the in-vitro, primer-directed, enzymatic amplification of nucleic acids

(Erlich *et al*., 1988; Saiki *et al*., 1988). This technique has been used in many diverse applications including diagnosis of plant diseases. For PCR, primers (small oligonucleotide probes) designed to anneal to specific DNA sequences in the target organism's chromosomal DNA or RNA, hybridize with and direct amplification of millions of copies of the target sequence. This amplified DNA can be visualized after electrophoresis in ethidium bromid estained agarose gels (Walcott, 2003). The remarkable proliferation of PCR-based methods for detecting pathogens in seeds has provided very useful tools that are available, and have begun to be implemented, in the vegetable seed industry and in some official seed testing laboratories for quality control testing (Agarwal, 2006).

Molecular techniques based on hybridisation or amplification, and especially on PCR, have been developed for the most important plant pathogenic viruses and bacteria. Although PCR can reach high sensitivity and specificity, its introduction for routine detection has been hampered by a lack of robustness (Van der Wolf *et al*., 2001). PCR has many beneficial characteristics that make it highly applicable for detecting seedborne pathogens. These include speed (completed within 2 to 3 h); specificity (DNA probes can be designed to amplify nucleic acids from the desired genus, species, subspecies, race, etc.); sensitivity (single copies of nucleic acids can be detected after amplification) and easy and objective result interpretation (the presence of a DNA fragment of specific size indicates the presence of the pathogen). Because of this great potential, over the past 10 years many PCR-based assays have been reported for seedborne pathogens (Prosen *et al*., 1993; Audy *et al*., 1996; Pasquini *et al*., 1998; Zhang *et al*., 1999; Hussain *et al*., 2000; Hadas *et al*., 2001; Frederick *et al*., 2002).

There are several obstacles that have slowed the adoption of PCR-based methods for seed health testing (Walcott, 2003). In the developing world, the capital costs and technical expertise for establishing PCR capabilities can be problematic. Even when costs and expertise are not major barriers, there can be technical impediments in terms of poor quality DNA and PCR inhibitors from seed extracts, leading to false negatives. Poor sensitivity also can result from low sampling intensity for PCR-based methods. One of the major obstacles to the adoption of nucleic acid–based seed health tests has been the potential for false positives due to the detection of remnant DNA from nonviable pathogen propagules (Agarwal, 2006).

PCR was initially considered to be too sensitive to be routinely applied as a seed health assay. There were also concerns about the ability of PCR to distinguish between dead and viable cells. Although PCR is a sensitive technique and, theoretically, capable of detecting a single bacterial cell, the sample size and volume of seeds (e.g., 30,000 seeds/liter of buffer) being tested in conjunction with the small volume (~4 µl) that can be used as template in the PCR reaction make PCR no more sensitive than many other techniques. As a consequence, other approaches, such as nested PCR (Ojeda and Verdier, 2000), have been used to detect pathogens in seeds. Nested PCR increases sensitivity by utilizing a second round of amplification using primers designed to anneal to internal regions of the amplicon produced by the first round of amplification. Using nested PCR, (Poussier *et al*., 2002). detected *Ralstonia solanacearum*, the causal agent of bacterial wilt in tomato seeds. Nucleic acid-based methods tend to be relatively expensive to apply and the advantages of these more rapid methods have to be considered against the less cost (but greater inconvenience to the grower, of the longer period required to achieve identification of a pathogen) of isolation-based methods. At present, a major disadvantage of nucleic acid methods in seed health testing is that of quantification. The technology is available and relatively simple to use. However, the cost of the necessary equipment so far remains prohibitive for use in routine seed health testing (Munkvold, 2009).

2.2.2.1. Use of nucleic acid–based methods in epidemiology research on seedborne pathogens

Methods used to detect pathogens in seeds can be valuable research tools for tracking the progress of the organisms during disease development. These methods can be applied to understand the sources of seedborne infections, the location of pathogens within seed tissues to confirm the occurrence of seed transmission and its mechanisms, and to understand the influence of external biotic and abiotic factors on seed transmission or other phases of the disease cycle. One example is watermelon fruit blotch (*A. avenae* ssp. *citrulli*), where the use of IMS-PCR facilitated the detection of a high incidence of infection in seeds from symptomless fruit following blossom inoculation (Walcott *et al*., 2003). This was the first indication that blossoms were an avenue of infection, in the absence of fruit blotch symptoms. Real-time PCR also was used to pinpoint the location of seedborne *A. avenae* ssp. *citrulli* infections to the surface of the perisperm-endosperm layer (Dutta *et al*., 2008). In rice, a BIOPCR technique was used to study survival of *Xanthomonas oryzae* pv. *oryza* in rice seed and track its progress in planta following seed transmission (Sakthivel *et al*., 2001). In olives, seed transmission of *Verticillium dahliae* was confirmed using a nested PCR assay in seedlings (Karajeh, 2006). One of the most interesting applications of nucleic acid–based methods in seed pathology has been the elucidation of embryo infection pathways for *Pea early browning virus* and *Pea seedborne mosaic virus* (PSbMV). Using a combination of approaches, including in situ hybridization, Maule and coworkers (Maule. 2007; Maule and Wang, 1996; Roberts *et al*., 2003) showed that the two viruses have different routes for embryonic infection. Whereas *Pea early browning virus* reaches the embryo as a result of gamete infection, PSbMV infects the developing embryo after fertilization. The PSbMV pathway is novel among viruses, and the results indicated a symplastic connection between maternal and filial tissues during embryo maturation (Maule, 2007).

2.2.2.2. Use of markers in seedborne pathogens

This is another approach that has made significant contributions to understanding the epidemiology of seedborne pathogens. For pathosystems involving ubiquitous pathogens or those with multiple infection pathways, marker use can be critical for differentiating seedborne strains from strains originating from other inoculum sources. The most commonly employed types of markers have been naturally occurring genetic markers, including antibiotic resistance, vegetative compatibility, and molecular markers unrelated to phenotype. Antibiotic resistance in bacterial pathogens, naturally occurring, induced through mutation, or inserted by genetic engineering, has been used effectively as a marker for decades (Munkvold, 2009).

Vegetative compatibility is a genetically controlled trait that describes the ability of fungal isolates to anastomose and form vegetative (asexual) heterokaryons. Strains that are vegetatively compatible are designated as members of the same vegetative compatibility group (Leslie, 1993). In fungi with a very diverse vegetative compatibility structure, the trait can be used to differentiate introduced strains from endemic strains. This approach has been used in a number of studies on seed transmission of *Fusarium verticillioides* and related *Fusarium* species. The approach commonly employs nitrogen utilization mutants (Klittich and Leslie, 1988) of the pathogen as a tool for recognizing compatible reactions. Conversely, using nitrogen-utilization mutants as marked strains, Galperin *et al*. (2003) concluded that seedborne inoculum was a significant source of inoculum for kernel infection of mature sweet corn plants. The contribution of seedborne inoculum to epidemics of Stagonospora leaf blotch was characterized by using *Stagonospora nodorum* strains identifiable by unique AFLP profiles (Bennet *et al*. 2007). Mycotoxin production is another genetic marker used to investigate the importance of seedborne *F. verticillioides*. Other markers have been employed by transforming pathogen strains with foreign genes that can act as molecular markers or easily distinguishable expression phenotypes. Two examples are genes for beta-glucuronidase (GUS) expression and fluorescent protein expression (Munkvold, 2009).

3. CONCLUSION

Seed is a small embryonic plant which is a basic unit of production for the world's food crop. It is an efficient means of introducing plant pathogens into a new area as well as providing a means of their survival from one cropping season to another. Seed health is a well recognized factor in the modern agricultural science for desired plant population and good harvest. Seed-borne fungi are one of the most important biotic constrains in seed production worldwide. ISTA, ISHI and NSHS are three primary organizations that publish standardized seed health test methods. Seed health testing to detect seed-borne pathogens is an important step in the management of crop diseases. Seed health is a measure of freedom of seeds from pathogens. Specificity, sensitivity, speed, simplicity, cost effectiveness and reliability are main requirements for selection of seed health tests methods. PCR has many beneficial characteristics that make it highly applicable for detecting seedborne pathogens. Some seed health testing methods can be applied to understand the sources of seedborne infections, location of pathogens within seed tissues, to confirm the occurrence of seed transmission and its mechanisms, and to understand the influence of external biotic and abiotic factors on seed transmission or other phases of the disease cycle. Since seed serve as means of dispersal and survival of plant pathogens, it is critical to test its health before using it as planting material. Seed health testing and detection is a first line approach in managing seedborn diseases of plants.

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