Detection of Seedborne Pathogens

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Summary. Plant pathogens present a serious threat to seedling establishment and the potential for plant disease epidemics under greenhouse conditions is great. Hence, pathogen exclusion by detection and elimination of infested seedlots remains a requisite tactic for seedling production and disease management. Unfortunately, the numbers of contaminated seed within a lot may be low and infested seed may be asymptomatic making their detection difficult. To address these issues seed detection assays have been developed, but many of them have shortcomings that reduce their effectiveness. Examples of frequently used seed assays include visual examination, selective media, seedling grow-out and serological assays which, while appropriate for some pathogens, often display inadequate levels of sensitivity and specificity. Recently, the polymerase chain reaction (PCR) has emerged as a tool for detecting microorganisms in many diverse environments. Thus far, it is clear that DNA-based detection systems exhibit higher levels sensitivity than conventional techniques. Unfortunately, PCR-based seed tests require the extraction of PCR-quality DNA from target organisms in backgrounds of saprophytic organisms and inhibitory seed-derived compounds. The inability to efficiently extract PCR-quality DNA from seeds has restricted the acceptance and application of PCR for seed detection. To overcome these limitations several modified PCR protocols have been developed including selective target colony enrichment followed by PCR (BIO-PCR) and immunomagnetic separation and PCR. These techniques seek to selectively concentrate or increase target organism populations to enhance detection and have been successfully applied for detecting bacteria in seed. Other techniques with great potential for rapid detection of seedborne pathogens include magnetic capture hybridization and PCR, and DNA-chip technology. Ultimately, PCR will be available for the detection of all seedborne pathogens and may supersede conventional detection methods.

Seedborne pathogens present a serious threat to seedling establishment. Close association with seeds facilitates the long-term survival, introduction into new areas and widespread dissemination of pathogens. Under greenhouse conditions, the risks of significant economic losses due to diseases are great because factors including high populations of susceptible plants, high relative humidity, high temperatures and overhead irrigation, promote explosive plant disease development. Under these conditions, the most effective disease management strategy is exclusion which is accomplished by using seed detection assays to screen and eliminate infested seedlots before planting. The following will explore the current state of seed detection technology and include recent advances. A summary of the features of each assay is presented (Table 1).

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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. While these tests have been used for many years, some of them have shortcomings that make them less than ideal. Brief descriptions of these assays including their advantages and disadvantages are discussed below.

Visual examination. In some cases infected seeds display characteristic symptoms, including discoloration and shriveling. Examples of such seedborne diseases include purple seed stain (*Cercospora kikuchii*) (Murakishi, 2002), and advanced stages of Phomopsis seed decay (*Phomopsis longicolla*) of soybean (*Glycine max*), and Cylindrocladium black rot (*Cylindrocladium parasiticum*) of peanut (*Arachis hypogeae*) (Randall-Schadel et al., 2001). In these cases seedlot infestation can be reduced by using automatic devices that sort seeds based on visual of physical characteristics (Paulsen, 2002; Walcott et al., 1998). These systems usually display low detection sensitivity. Additionally, seeds infested by fungi, bacteria and viruses may display no macroscopic symptoms, making visual or physical inspection of seeds useless as a detection assay.

Selective media. A direct method of testing seeds is by allowing pathogens to grow from them onto appropriate artificial media. This can be done by directly plating surface-sterilized seed samples or seed-wash liquid onto artificial media, followed by incubation under adequate conditions. Once a pathogen is isolated it can be identified by its cultural or biochemical characteristics e.g. the production of a bluish-green fluorescent pigment on King's B medium (King et al., 1956) in the case of fluorescent *Pseudomonas* spp. or the production of dark, muriform conidia in the case of *Alternaria* spp. Unfortunately, seeds may be contaminated by saprophytic microorganisms (nonpathogens) that grow as well as, or better than target organisms on nutrient-rich, artificial media. The excessive growth of saprophytic organisms including *Rhizopus* spp., *Penicillium* spp., and yeasts make it impossible to identify pathogens that may be present. The inability to identify the unique characteristics of the target pathogens in the presence of contaminating microorganisms lead to inaccurate assessments of seedlot infestation. To overcome this problem, selective artificial media are developed that use antibiotics, fungicides, selected carbon and nitrogen sources and other inhibitory compounds to retard the growth of nontarget microflora while allowing the pathogen to grow. Many selective and semiselective media have been developed for seedborne fungi and bacteria (Chang et al., 1991; Franken et al., 1991; Kritzman and Netzer, 1978; Lorbeer and Tichelaar, 1970; Toussaint et al., 2001). Unfortunately, development of such media is time consuming and requires specific knowledge of the nutritional requirements and chemical tolerances of the target organism, relative to the nontarget seed microflora. Employing selective media also requires 2 to 4 d for pathogen growth and the test operator must be familiar with the range of cultural characteristics associated with the pathogen. Finally, while selective media can be applied for certain bacteria and fungi, it cannot be applied for nonculturable obligate parasites, e.g., viruses, nematodes and certain fungi and bacteria.

Serology-based assays

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. 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 assays do not require pure isolations of the pathogen and, hence, are applicable to biotrophic and necrotrophic seedborne pathogens. Currently serology is the most widely used detection assay for seedborne viruses and it has proven to be sensitive and robust (Barba, 1986; Barba, 1986; Bossennec and Maury, 1978; Delecolle et al., 1985; Falk and Purcifull, 1983; Pasquini et al., 1998). Serology has also been widely used for the detection of bacterial and fungal plant pathogens, but the unavailability of species-specific antibodies is a limitation (Franken et al., 1993; Frommel and Pazos, 1994; Higley et al., 1993; Linfield et al., 1995; McLaughlin and Chen, 1990; Rajeshwari et al., 1998;). Additionally, the detection thresholds of serology-based assays vary significantly based on the quality of the antibody and the testing format. Finally, with serology-based assays it is possible to detect nonviable pathogens which results in erroneous (false-positive) interpretation.

Seedling grow-out assay

The seedling grow-out assay is a direct measure of the the seedlot's ability to transmit a disease. To conduct this assay, seedlot samples are planted under greenhouse conditions conducive to disease development and after germination, seedlings are observed for the development of symptoms. Seedling grow-out is one of the most applicable and widely used seed detection assays (Capoor et al., 1986; Lee et al., 1990; Yang et al., 1997) but for successful implementation, infected seedlings must display obvious and characteristic symptoms. Unfortunately, this is not always the case as some diseases have nondistinct symptoms, e.g., wilting, chlorosis etc. Another drawback of the seedling grow-out assay is that large seed samples (10,000 to 50,000 seeds in the case of bacterial fruit blotch (*Acidovorax avenae* subsp. *citrulli*) of watermelon (*Citrullus lanatus*) must be tested to statistically ensure that one infested seed can be detected. In addition to losses associated with the destructive testing of expensive seeds, assaying this quantity of seeds requires large areas of greenhouse space and adequate labor for assay set up and evaluation. The seedling grow-out assay is also

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 specificity	Time 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)	3–4 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	40–60 min	Very high	Complicated, expensive	Very high
DNA microarrays	6 h	Very high	Complicated; very expensive	Very high

time consuming, requiring up to 3 weeks for seedling germination and symptom development. Finally, seed test evaluators must be familiar with the symptoms associated with each disease. This can be difficult since each disease has a range of possible symptoms that are influenced by environmental conditions. Hence, for the seedling grow-out assay, greenhouse conditions must be strictly regulated to ensure consistent results. In large greenhouses this can be a challenge and it can lead to erroneous test results. Also, because of the variations in seedling symptom expression it is often necessary to isolate the pathogen from suspected seedlings for confirmation. These extra steps further prolong the time required to complete the seedling grow-out assay. Residual contamination and cross-contamination between spatially separated seedlots are also issues of concern under greenhouse conditions.

Polymerase chain reaction-based seed detection assays

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 bromide-stained agarose gels. 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 (Audy et al., 1996; Frederick et al., 2002; Hadas et al., 2001; Hussain et al., 2000; Pasquini et al., 1998; Prosen et al., 1993; Zhang et al., 1999). Despite the potential improvements over conventional assays, PCR-based seed assays have not been widely adopted by commercial and government testing agencies in the U.S. One reason for this lack of acceptance is that many seed types contain compounds (e.g., tannins, phenolic compounds, phenolic compounds) that inhibit DNA amplification resulting in false-negative results when PCR is attempted directly on seed extracts. To circumvent the inhibitory effects of seed compounds, elaborate DNA extraction and purification steps are employed, that not only reduce assay sensitivity and efficiency, but also require the use of potentially harmful chemicals e.g. chloroform and phenol. Finally, DNA from nonviable cells or tissues can yield false-positive results in seed assays, so it is necessary to confirm positive results by recovering the target organism. Management decisions based on these types of errors may result in the unnecessary destruction of healthy seedlings and subsequent financial losses for commercial seedling producers.

To unlock the invaluable potential of PCR for detecting seedborne pathogens, several modifications have been developed to specifically overcome the above-mentioned limitations. These include enrichment or BIO-PCR, immunomagnetic separation (IMS) and magnetic capture-hybridization (MCH). The following discussion will describe these techniques.

BIO-PCR. Target cell enrichment followed by PCR or BIO-PCR improves the efficiency and sensitivity of PCR by allowing target pathogen populations to increase in a preenrichment phase, before DNA extraction and PCR. Selective preenrichment increases pathogen populations relative to nontarget microorganisms and results in higher quantities of target DNA, which ultimately results in higher sensitivity. Additionally, during incubation and enrichment on artificial media, inhibitory compounds are adsorbed or diluted during cell harvest, and do not interfere with DNA amplification. For this technique, seed samples are washed or crushed in an appropriate buffer to extract seedborne

Comments	Applicability for Fungi	Bacteria	Viruses
Relies on presence of symptoms on seeds that may not always be present, low reliability	X	X	X
Many good selective media exist but they may be difficult to develop	X	X	
Requires large areas of greenhouse space and ability to recognize symptoms	X	X	X
Very reliable and widely used for viruses; however, sensitivity and specificity is of concern for bacteria	X	X	X
Affected by inhibitors in seeds; lengthy DNA extraction procedures are required	X	X	X
Cannot be applied to obligate parasites	X	X	
Highly sensitive but requires polyclonal antibodies	X	X	X
Applicable to all pathogens but subject to false positives due to detection of DNA from nonviable pathogens	X	X	X
While actual PCR time is short, DNA extraction is still required	X	X	X
Will allow simultaneous testing for multiple pathogens; many of the same limitations as PCR	X	X	X

bacteria. Aliquots of the seed wash are spread onto semiselective media and incubated for 2 to 3 d. Colonies are then harvested and after DNA extraction, PCR is conducted with specific primers. In the case of seedborne fungi, seeds are incubated under conditions of high relative humidity to increase target fungus mycelial mass before DNA extraction and PCR (Pryor and Gilbertson, 2001).

BIO-PCR has been developed for the detection of bacterial fruit blotch of watermelon, halo blight (*Pseudomonas syringae* subsp. *phaseolicola*) of beans (*Phaseolus vulgaris*), bacterial ring rot (*Clavibacter michiganensis* subsp. *sepidonicum*) of potato (*Solanum tuberosum*) and black rot (*Alternaria radicina*) of carrot (*Daucus carota*) (Pryor and Gilbertson, 2001; Schaad et al., 1995, 1999). This technique has been reported to significantly improve the sensitivity and the ease of implementation of PCR, displaying detection limits of 2 to 3 cfu/mL (Schaad et al., 1999). The advantages of BIO-PCR have also been demonstrated in a comparison between direct PCR, double antibody sandwich ELISA and semiselective media (Wang et al., 1999) for the detection of *Xanthomonas albilineans,* the causal agent of leaf scald of sugar cane (*Saccharum* spp.). Finally, with BIO-PCR the target organism must grow on the selective medium before it can be detected by PCR. Hence, only viable colonies are detected as opposed to DNA from nonviable cells.

The disadvantages of BIO-PCR include the need for a semiselective medium for each pathogen. As mentioned earlier, semiselective media require specific knowledge about the nutritional requirements and chemical tolerances of the target organism and usually take time to develop. Fortunately, in the case of BIO-PCR, there is no need to identify the pathogen based on colony morphology since specific PCR primers are used. As such it is only necessary for the selective media to retard the growth of nontarget organisms. BIO-PCR also requires two to three days for bacteria and 5 to 7 d for fungi to grow, significantly increasing the time required for assay completion. Another critical drawback of BIO-PCR is that it cannot be used for obligate parasites (e.g., viruses). As such, it is limited primarily to readily culturable bacteria and fungi.

IMMUNOMAGNETIC SEPARATION AND PCR (IMS-PCR). Immunomagnetic separation refers to the use of microscopic magnetic beads (IMBs) coated with antibodies produced against a specific microorganism, to selectively sequester target cells from suspensions containing heterogenous cell mixtures (Olsvik et al., 1994; Safarik and Safarikova, 1999). Captured cells can be recovered on selective media or DNA can be extracted from them and used for PCR. This technique has been employed widely for the detection of microorganisms from a variety of backgrounds including food, and feces (Vernozy-Rozand et al., 1997; Widjojoatmodjo et al., 1992). Recently IMS-PCR was developed for the detection of *A. avenae* subsp. *citrulli* in watermelon seeds (Walcott and Gitaitis, 2000). Bacterial fruit blotch poses a perennial threat to the US watermelon seed and transplant industry (Latin and Hopkins, 1995). To use IMS, seeds are washed or crushed in buffer and seed debris is removed. Bacteria in the seed wash are pelleted by centrifugation and resuspended in buffer. IMBs coated with specific antibodies are incubated with the seedwash, during which time the antibodies bind target bacteria. After immunocapture, the IMBs are immobilized with a magnet and washed thoroughly to eliminate inhibitors and nontarget bacteria. IMBs can then be spread onto selective agar medium and incubated until bacterial colonies can be observed. Alternatively captured bacteria can be lysed by boiling to release DNA that can be used for PCR.

IMS-PCR significantly improves the detection efficiency and sensitivity over conventional PCR (Walcott and Gitaitis, 2000). IMS consistently recovered viable target colonies from suspensions containing 10 target cfu/mL. Additionally, at least 10-fold more CFUs were recovered by IMS than by direct spread-plating. The frequency at which IMS-PCR could detect suspensions with 10 target cfu/mL was 43%, however this improved to 83% for suspensions with 10,000 cfu/mL. Finally, IMS-PCR proved to be more sensitive and reliable than hexacetyldimethylethyl ammonium bromide (CTAB)-DNA extraction (Ausubel et al., 1987) followed by direct PCR and ELISA for the detec-

tion of *A. avenae* subsp. *citrulli* in watermelon seedlots. IMS-PCR also facilitated the detection of *A. avenae* subsp. *citrulli* in seedlots with 0%, 1%, 5%, and 10% (Walcott and Gitaitis, 2000). Using samples of the same seedlots, CTAB-DNA extraction followed by PCR and ELISA failed to detect the bacterium. To date, in addition to *A. avenae* subsp. *citrulli*, an IMS-PCR-based seed assay has been reported for center rot of onion (*Allium cepa*) caused by *Pantoea ananatis* (Walcott et al., 2002).

Despite its many advantages, however, IMS-PCR is limited by the fact that it relies on polyclonal antibodies for specific capture of organisms. Polyclonal antibodies are not readily available and must be produced for each pathogen. Additionally, since IMS-PCR relies on microscopic beads for pathogen capture, it is unlikely that they would be effective at recovering filamentous mycelia of plant pathogenic fungi.

MAGNETIC CAPTURE HYBRIDIZATION AND PCR (MCH-PCR). Magnetic capture hybridization and polymerase chain reaction is similar in format to IMS-PCR. The techniques differ however, in that MCH-PCR uses single-stranded DNA probes to capture and concentrate specific DNA fragments that can then be used as templates for PCR. MCH-PCR is a relatively new technique, first described in 1995 for the detection of *Pseudomonas fluorescens* in nonsterile soil (Jacobsen, 1995). This technique has subsequently been developed for the detection of fungi, viruses and bacteria in water, wood, soil and food, all of which contain PCR-inhibitory compounds (Chen and Griffiths, 2001; Chen et al., 1998; de Moraes et al., 1999; Langrell and Barbara, 2001). Despite its great potential as a detection assay, MCH-PCR has not been applied for the detection of seedborne pathogens. Recently, we have initiated research to develop a MCH-PCR assay for the detection of *Botrytis aclada* (causal agent of *Botrytis* neck rot) on onion seed. This economically important disease of onion causes significant postharvest economic losses and there is evidence of a strong relationship between seed borne inoculum postharvest losses (Maude, 1983). Speculation about the role of *B. aclada*-infested seedlots in recent neck rot outbreaks in New York has renewed interest in the epidemiological significance of seed borne inoculum. This has prompted the search for more accurate and sensitive seed assays. Currently, the standard assay for *B. aclada* involves plating seeds on semiselective media, followed by a 7 to 10 d incubation and subsequent observation of the morphological characteristics of isolated fungi (Lorbeer and Tichelaar, 1970; Kritzman and Netzer, 1978). While polyclonal antibodies have been developed for *B. aclada* (Linfield et al., 1995), there have been no reports of attempts to use them to test seeds.

For MCH-PCR, specific oligonucleotide primers, Ba1r/Ba2f, designed based on random amplified polymorphic DNA data (Nielsen et al., 2002) were used. These primers directed amplification of a 413 base pair (bp) amplicon, one strand of which was used to design a complementary 117 bp, single-stranded DNA capture probe. The capture probe was synthesized with biotin attached to its 5'-prime end (The University of Georgia, Molecular Genetics and Instrumentation Facility, Athens) and attached to microscopic, streptavidin-coated magnetic beads (Dynal Oslo, Norway). To conduct MCH-PCR, infested onion seeds were crushed and DNA was extracted using a Mini-Beadbeater (Biospec Products Inc., Bartlesville, Okla.). Double-stranded target DNA molecules were denatured by boiling and magnetic beads coated with the capture probe were incubated with the DNA suspensions for 2 h at 62 °C (143.6 °F). During this time, the capture probe hybridized with single-stranded target DNA fragments. After incubation, the beads were washed and the captured DNA was used for PCR.

Development of the MCH-PCR-based seed detection assay is still in progress, however, we have observed detection thresholds of 10 conidia/mL in the presence of PCR-inhibiting onion seed wash. Additionally, MCH-PCR displayed a detection threshold of 10^{-13} g·mL^{-1} of *B. aclada* DNA (Walcott, unpublished data). Finally, the applicability of MCH-PCR for detecting *B. aclada* in naturally infested seelots is currently being determined but thus far, positive results have been obtained for seedlots with 1% infestation (determined by plating on selective agar media). It is clear that MCH-PCR has great potential as a seed detection assay. The benefits of this assay include applicability to all pathogens; rapidity, since it can be completed within a day; and the ability to overcome the inhibitory effects of seed compounds. In contrast to IMS-PCR, DNA capture probes can be readily synthesized and may be more accessible than polyclonal antibodies. Hence, this technique may be more widely implemented. Unfortunately, MCH-PCR captures and detects DNA as opposed to viable pathogen propagules and it would be impossible to determine whether amplified DNA originated from viable or nonviable organisms. However, this potential problem could be solved by targeting unique mRNAs for detection. Subsequent reverse transcription PCR could be used for amplification and detection of target DNA. Since mRNA is produced only in viable cells, and degrades rapidly, this would confirm the detection of only viable organisms and cells. However, this approach would be more technically challenging and more costly.

RAPID-CYCLE REAL-TIME PCR. As previously mentioned, commercial and government seed testing agencies have been slow to adopt PCR-base seed detection assays. This has been due in part to the cost of the equipment and consumables, and level of technical expertise required to conduct the assay. Additionally, the risks of cross-contamination and the need for post-PCR steps such as gel electrophoresis, have made the technique unattractive. Recent advances in PCR, in the form of rapid-cycle real-time PCR promise to eliminate many of these barriers and make PCR more accessible for seed detection. With real-time PCR, DNA amplification is coupled with the production of a fluorescent signal that increases proportionally with the numbers of amplicons produced (Kurian et al., 1999; Cockerill and Smith, 2002). The fluorescent signal is monitored on a computer in real-time and provides an indirect visual representation of DNA amplification. Detection of amplified DNA can be accomplished by staining with SYBR Green I (Molecular Probes Inc., Eugene Ore.) that binds double-stranded DNA indiscriminately or with the use of specific reporter probes like TaqMan (Applied Biosystems, Foster City, Calif.) (Taylor et al., 2002). TaqMan probes are synthesized with reporter and quencher

dye molecules at the 5' and 3' ends respectively. In this configuration (reporter proximal to the quencher dye), there is no fluorescence but when they are separated the reporter dye fluoresces. With the TaqMan system, the first step in PCR is the annealing of a complementary probe to the template DNA. Taq DNA polymerase has 5' exonuclease activity (cleaves off nucleotides from the 5' end of nontemplate complement DNA) and during the extension step of PCR, the TaqMan probe is excised, separating the reporter dye from the quencher molecule. This results in fluorescence that is detected by photosensors. The intensity of fluorescence is directly related to the excision of reporter dye molecules, which is directly related to DNA amplification. Other detection systems including fluorescent resonance energy transfer (FRET) and molecular beacon probes are also employed for real-time PCR (Cockerill and Smith, 2002).

As compared to conventional PCR, real-time PCR has several key advantages that potentially make it more acceptable for use in routine seed testing. These include 1) rapid cycling which reduces DNA amplification time significantly, 2) completion of PCR in a closed system which reduces the risk of cross-contamination i.e. DNA amplification and subsequent DNA detection is accomplished in the same tube; 3) there is no need for time consuming post-PCR electrophoresis to determine PCR results; 4) the use of different dyes and probes can allow for mutliplex PCR, by which multiple pathogens can be detected in the same reaction (Wittwer et al., 2001) and 5) real-time PCR can allow quantification of template DNA which may be of use in determining levels of seed infestation. On the other hand, there are some key factors that may prevent the immediate adoption of this technology for seed detection. These include the facts that real-time PCR requires thermal cyclers that are equipped to detect fluorescence. These thermal cyclers are significantly more expensive than conventional thermal cyclers and TaqMan probes are costly. Finally, despite the advantages listed above, real-time PCR is subject to many of the problems that hamper conventional PCR , including inhibition by seed-derived compounds. Hence, it is still necessary to implement strategies upstream of PCR that produce PCR-quality template DNA/RNA. Significant benefits can be realized by combining real-time PCR with the other PCR modifications mentioned above (MCH-PCR, IMS-PCR, BIO-PCR). To date, real-time PCR seed detection assays have been reported for *A. avenae* subsp. *citrulli* in watermelon seeds (P. Randhawa, personal communication) and *Microdochium nivale* in wheat (*Triticum* spp.) seeds (Taylor et al., 2002). It is likely that more real-time PCR seed assays will be developed as the technology becomes more affordable.

DNA Chip (microarray) technology

DNA chips or microarrays represent another DNA-based detection assay that may be applied to test seeds for pathogens. This relatively new technology relies on the unique ability of nucleic acid molecules to hybridize specifically with molecules with complementary sequences (Lemieux et al., 1998; Vernet, 2002). With DNA chip technology, oligonucleotide probes are attached to small (approximately 1 cm^2) glass or silica-based surfaces (chips). The power of this technique lies in the fact that hundreds to thousand of oligonucleotides can be attached to specific, locations on each chip. These oligonucleotides can be complementary to DNA sequences that are unique to certain microorganisms and hence, can be used detect pathogens in seed samples. To apply DNA-chip technology, DNA or RNA must be extracted from the sample being tested and amplified. The amplified DNA is digested into smaller fragments that are then labeled with fluorescent markers and hybridized with oligonucleotides fixed to the DNA chip. After hybridization, the chip is washed thoroughly and fluorescence, which is directly proportional to the amount of nucleotide retained, is measured. If the DNA from the pathogen of interest in present in the seed sample, then the oligonucleotide probe at the position on the chip that corresponds to that pathogen will display fluorescence.

DNA-chips are being used in many different fields for diagnosis (Anthony et al., 2000; Lemieux et al., 1998). Advantages of this technology include simultaneous detection of a wide range of pathogens and rapid completion time (6 h). However, since DNA-chip technology relies in part, on DNA amplification, it has similar limitations as those described for conventional PCR. Additionally, significant technological expertise and expensive equipment are required. Currently, few DNA-chip seed detection assays have been developed (J. van der Wolf, personal communication; Fessehaie et al., 2001). However, it is envisioned that this technology will be more widely employed for routine seed testing in the future.

Conclusions

The environmental conditions in seedling establishment systems are usually highly favorable for disease development. Therefore, it is critical to ensure that no potentially damaging pathogens are introduced on seeds. This can most effectively be accomplished by exclusion, using seed detection assays to identify contaminated seedlots that can then be discarded or treated. Conventional seed detection assays including visual examination, selective media, serological assays and the seedling grow-out assay have been used extensively, but all have shortcomings ranging from inefficiency to lack of specificity and sensitivity. PCR holds great potential for improving pathogen detection in seeds, as it embodies many of the key characteristics including specificity, sensitivity rapidity, ease of implementation and interpretation and applicability. While inhibitory seed compounds can limit the applicability of conventional PCR, modifications including BIO-PCR, IMS-PCR and MCH-PCR may provide opportunities to circumvent inhibitory compounds while improving detection of seedborne pathogens. IMS-PCR and MCH-PCR are particularly attractive because they provide simple and universally applicable formats for testing seeds for different culturable and nonculturable pathogens. Further improvements in the cost and efficiency will eventually allow DNA-based detection systems to replace the vast array of seed detection assays currently employed and provide superior detection capabilities necessary for healthy seedling establishment.

Like other fields in which pathogen detection is critical, seed detection assays must be based on new technologies. However, before adopting these assays, it is critical to rigorously evaluate their applicability, precision, and

accuracy in real-world, high throughput testing of naturally infested seeds. There are many reports of new seed detection assays in the scientific literature, however, few of these are developed past the initial stages. Hence, little is known about their applicability for routine seed testing. To ensure that these assays work, they must be validated in stringent multilaboratory tests which evaluate their reproducibility and repeatability. Only assays evaluated in this manner should be considered for testing of commercial seeds.

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