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REVIEW

Diagnostic methods for detecting fungal pathogens on vegetable seeds

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Early diagnosis of seedborne fungal pathogens is particularly important, as often, infected seeds appear symptomless; seed diagnosis can avoid uncontrolled propagation of pathogens through long-distance exchange of such material. This will prevent economic losses and unnecessary use of fungicides, so reducing costs and the introduction of toxic substances into the environment. Traditional techniques for detection of seedborne fungi are based on incubation and grow-out methods. Although these are frequently used because of their simplicity of application, they are time-consuming, require mycological skills, and are sometimes not sensitive enough to low levels of seed infection. Recently, new identification techniques, based on DNA analysis, have been applied and are very efficient due to high sensitivity and specificity. The most common technique is conventional PCR, while other recent techniques include nested PCR, to obviate low levels of target pathogens, multiplex PCR, to detect several pathogens simultaneously, real-time PCR, to quantify fungi on seeds, and magnetic-capture hybridization PCR. The main drawbacks of molecular methods are the inability to distinguish between vital and non-vital inocula, and the difficulty in obtaining quality DNA template, due to PCR inhibitors in seeds. To reduce inhibitory effects, several modified PCR protocols, such as loop-mediated isothermal amplification, and non-destructive testing methods have been developed. Loop-mediated isothermal amplification and next-generation sequencing have been widely applied in nucleic acid analysis and, given the numerous advantages provided, their application can be substantially extended in the future for detection of fungal pathogens in seeds.

Keywords: Alternaria spp., *Botrytis* spp., diagnosis, PCR, seedborne fungi

Introduction

The movement of plant germplasm and any other raw plant material can pose great risks for the spread of diseases and pests (Gergerich *et al.*, 2015). In particular, seeds represent a particularly efficient vehicle to disperse seedborne pathogens. Unlike infected vegetative plant tissues, infected seeds are in most cases symptomless; additionally, low percentages of seed infection can result in severe crop losses (Pellegrino *et al.*, 2010), particularly if the pathogens are quarantine microorganisms.

The close association of fungi with seeds facilitates long-term survival and widespread dissemination of such pathogens. Many countries have formulated legislation that helps to limit or prevent the introduction of exotic pathogens into new areas, and these are generally supported by detection techniques. However, the low inoculum levels and the often non-uniform distribution within seed lots make the testing of seeds for pathogens a difficult task.

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The detection approaches that are applied to fungal seed pathogens have been developed using different technologies. However, few of these can satisfy the minimum requirements for adequate seed testing. Over the last few decades, the advent of serological and molecular tools has promoted efforts to set up assays with specific technical aspects (e.g. specificity, sensitivity, robustness) and economic demands (e.g. short diagnosis time, high-throughput, minimum taxonomic expertise, minimum cost; Walcott, 2003; Lievens & Thomma, 2005).

Considering that accurate and rapid identification of these organism(s) is essential to satisfy phytosanitary regulations, and consequently for effective disease control, the present review outlines and critically discusses the traditional and innovative diagnostic techniques that have been applied to seedborne fungal pathogens.

Conventional Fungal Detection Assays

Visual examination

Some fungal pathogens can cause symptoms on seeds that are visible to the naked eye, or at least under low magnification (Chen *et al.*, 2007). Sometimes symptoms can be attributed to certain fungal species, such as discolouration, shrivelling and cracks on soybean seeds that

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are typical of *Phomopsis* spp. and *Cercospora kikuchii* (Murakishi, 2002; Li, 2011), and the speckles on peanut seeds that are caused by *Cylindrocladium parasiticum* (Randall-Schadel *et al.*, 2001).

In most cases, identification of a pathogen based only on such symptoms is not recommended, as these can be common to several fungi (Andersen & Leach, 1961). Examination of seeds under a stereoscopic microscope allows detection of the presence of overwintering or reproductive structures of fungi, such as the crust of oospores of *Peronospora manshurica* on soybean seeds (Agarwal *et al.*, 2006), the acervuli and microsclerotia of *Colletotrichum dematium* on chilli seeds (Kumar *et al.*, 2004), and the pycnidia of *Septoria apii* embedded in the seed coat of celery seeds (Horst, 2008). However, when seeds that are infected by fungi do not show macroscopic symptoms, no sort of visual inspection has a rational basis as a detection assay (Walcott, 2003).

Seed washing techniques can be used in combination with visual examination and are useful for testing for surface-borne pathogenic fungi (Rao *et al.*, 2006), or when seedborne fungi are present as microscopic spores on the seed surface (Agarwal *et al.*, 2006). These techniques allow the removal of spores, hyphal elements, or other fungal structures from the seeds by washing and stirring them in distilled water (Reeves, 1998). Following the washing, the resulting suspension can be decanted and examined under a stereomicroscope, or the pellet, obtained after a centrifugation, can be examined under a compound microscope. The number of spores for a known amount of seed can also be determined microscopically with a haemocytometer counting chamber (Andersen & Leach, 1961).

Incubation methods

Incubation methods are the most frequently used technique for detection of seed-transmitted pathogens, especially for high-incidence fungi that can occur on seed samples at a level >1%, also providing information about the viability of the inoculum on the seeds (Marcinkowska, 2002). Seeds can be incubated in Petri dishes that contain medium-impregnated filter paper (i.e. blotting) or different agar media, under conditions that will promote fungal growth and sporulation. Such samples are generally exposed to light–dark cycles of 12/12 h. The times and temperatures for plate incubations vary depending on the fungal species, but generally this ranges from 2 to 10 days, with a temperature from 20 to 28 °C (Shovan *et al.*, 2008; Elwakil *et al.*, 2009; Naqvi *et al.*, 2013). A standard sample for testing uses 400 seeds (Marcinkowska, 2002; Rao *et al.*, 2006), although this will depend on the seed size.

Saprophytic microorganisms can often be present on seed surfaces. To limit or eradicate them, the seeds can be surface sterilized if the pathogen to be detected is inside the seed (El-Nagerabi & Elshafie, 2000; Du Toit *et al.*, 2005; Rodrigues & Menezes, 2005). Different surface disinfectants can be used for this, such as 0.5–1.0% sodium hypochlorite, which is the most common (Roy *et al.*, 2000; Peres *et al.*, 2002), 0.1% mercuric chloride (El-Nagerabi & Elshafie, 2000; Ora *et al.*, 2011), or 10–90% ethanol (Vallad *et al.*, 2005).

In blotting tests, low temperatures or chemicals can be applied to prevent seed germination, which can interfere with the growth of the seedborne fungi; these treatments disrupt the vitality of the seed tissues (Ha *et al.*, 2009). For example, the sodium salt of the herbicide (2,4-dichlorophenoxy) acetic acid monohydrate (2,4-D) is often used to moisten the filter paper in blotting tests (Kumar *et al.*, 2004; Alves & Pozza, 2009); the freeze blotter test includes an additional step where seeds are left to soak for 24 h and then kept at −20 °C for 24 h to kill the embryo (Du Toit *et al.*, 2005; Elwakil *et al.*, 2009).

The agar media most commonly used for incubation tests are the non-selective potato dextrose agar and malt extract agar (Roy *et al.*, 2000; Marcinkowska, 2002; Nasir, 2003; Ora *et al.*, 2011). However, fungal reproductive structures are not always produced on these substrates. To stimulate fungal sporulation, a medium that is less rich in nutrients is more advisable, such as water agar or cornmeal agar (Roy *et al.*, 2000; Afouda *et al.*, 2009).

Acidified agars and selective and semiselective media can be used to reduce bacterial contaminants (Pyndji *et al.*, 1987) and saprophytic microorganisms, which can prevent the growth of the pathogens. Many selective and semiselective media have thus been developed for specific seedborne fungi, through the addition of antibiotics, such as streptomycin sulphate (Pellegrino *et al.*, 2010) and penicillin G (Peres *et al.*, 2002; Nasir, 2003); fungicides, such as benomyl (Soteros, 1979); other specific chemicals, such as bromophenol blue (Peres *et al.*, 2002); or inhibitory compounds (Marcinkowska, 2002; Walcott, 2003). A selective medium has been developed specifically for *Fusarium oxysporum* that reduces the development of fungal contaminants and allows *F. oxysporum* colonies to grow rapidly (Komada, 1975; Chiocchetti *et al.*, 1999; Vannacci *et al.*, 1999; Garibaldi *et al.*, 2004). Selective media are generally more practical for fungal detection, which then do not require further steps for their identification.

At the end of these incubation periods, the seeds are individually examined under a stereomicroscope (Cappelli & Covarelli, 2005; Alves & Pozza, 2009) and the fungal colonies that have developed on agar media can be identified according to macroscopic and microscopic features (Peres *et al.*, 2002; Pryor & Gilbertson, 2002; Ora *et al.*, 2011).

The blotter and agar-plate methods are simple and inexpensive ways to detect seedborne fungi that respond by sporulation (Rao *et al.*, 2006). However, the blotter test does not always provide adequate conditions for the development of mycelial growth, sporulation, or for the symptoms of the pathogen to appear on the seeds. For these characteristics, it is preferable to incubate the seeds on agar media, where as well as developing into characteristic colonies, greater numbers of fungal colonies can be formed (Marcinkowska, 2002; Nasir, 2003).

Serological Fungal Detection Assays

Immunological methods for the detection of seed-transmitted pathogens are based on the use of polyclonal or monoclonal antibodies that specifically bind to a target antigen, allowing the pathogen to be subsequently detected by enzymatic conversion of substrates or using fluorescent tags. The immunological method most commonly used is the double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA; Ward *et al.*, 2004). Serological assays do not require pure isolation of the pathogen, and hence they are applicable to biotrophic as well as necrotrophic seedborne pathogens. These assays are widely applied to detect seedborne viruses, but less so for fungal plant pathogens, due to the lack of species-specific antibodies. Furthermore, serology-based assays can also detect non-viable pathogens, which can result in erroneous interpretations (Walcott, 2003; Afouda *et al.*, 2009).

Molecular Fungal Detection Assays

Conventional PCR

PCR has numerous positive characteristics, including rapidity, specificity, sensitivity and easy interpretation, which make it suitable for detection of seedborne pathogens (Walcott, 2003; Ward *et al.*, 2004).

As a result of this great potential, over the past 20 years, many PCR-based assays have been reported for the identification of seedborne pathogens; e.g. *Ascochyta lentis* from lentil seeds, *Alternaria radicina* from carrot seeds, *Alternaria brassicae* in cruciferous seeds, *Leptosphaeria maculans* from canola seeds, and *Phoma valerianella* from lamb's lettuce seeds (Hussain *et al.*, 2000; Pryor & Gilbertson, 2001; Guillemette *et al.*, 2004; Landa *et al.*, 2007; Chen *et al.*, 2010; Pellegrino *et al.*, 2010). PCR-based methods also provide rapid and unequivocal identification of *F. oxysporum* f. sp. *basilici* from basil seeds, unlike the conventional detection methods that cannot distinguish between pathogenic and non-pathogenic *F. oxysporum* isolates (Baayen, 2000; Chiocchetti *et al.*, 2001).

One of the most important advantages that molecular-based detection techniques have over conventional diagnostic methods is the ability, in principle, to distinguish between closely related organisms. However, the presence of compounds within the seeds that can inhibit DNA amplification, resulting in false negatives, can be a problem. To overcome these limitations, several modifications have been developed, starting from DNA extraction, to the amplification of the DNA.

The DNA recovered from seeds after phenol-chloroform extraction and ethanol precipitation often contains PCR inhibitors that cannot be removed by repeated washing with 70% ethanol or by repeated DNA precipitation. Dilution of DNA extracts has been shown to eliminate the effects of PCR inhibitors, but it also reduces the PCR sensitivity (Demeke & Jenkins, 2010). To overcome this problem, the cetyl trimethylammonium bromide (CTAB) method can be applied with the addition of particular chemical and enzymatic treatments (Terry *et al.*, 2002). To remove polysaccharides and proteins, treatments with enzymes such as pectinase, cellulase, hemicellulose, α-amylase, proteinase K and glycoside hydrolases can be used (Rether *et al.*, 1993; Demeke & Jenkins, 2010). Alternatively, the high levels of polysaccharides in soybean and chickpea seeds during DNA extraction can be precipitated using high concentrations of NaCl and Sarcosyl (Sharma *et al.*, 2002). There are several other methods for reducing the effect of PCR inhibitors. A silica matrix purification can be used instead of ethanol precipitation to recover the DNA. The DNA recovered from lettuce seeds using a silica matrix does not contain the brown pigments that are responsible for inhibition of DNA amplification, and the DNA of the target fungus, *F. oxysporum* f. sp. *lactucae*, can be consistently amplified without the requirement for further dilution (Demeke & Jenkins, 2010; Mbofung & Pryor, 2010). Commercial DNA extraction kits can also efficiently remove most PCR inhibitors (Ma & Michailides, 2007); this has allowed the detection of several fungal pathogens from seeds, such as *Ascochyta rabiei* from chickpea seeds (Phan *et al.*, 2002), *L. maculans* from canola seeds (Chen *et al.*, 2010), and *Botrytis* spp. from onion seeds (Chilvers *et al.*, 2007). However, their cost is higher than that of CTAB methods.

Reagents can be added to PCR mixture buffers, to allow consistent amplification of the target DNA fragment from undiluted DNA extracts from seeds. To detect *A. radicina* on carrot seeds efficiently, Pryor & Gilbertson (2001) reported that the addition of 0.2% skimmed milk in the PCR mixture allowed consistent amplification of the target fungal DNA from the undiluted DNA obtained from infected seeds. Bovine serum albumin improves the sensitivity for the detection of *F. oxysporum* f. sp. *basilici* from basil seeds (Pasquali *et al.*, 2006) and *Alternaria alternata* and *A. radicina* from infected carrot seeds (Konstantinova *et al.*, 2002). The use of 8% glycerol has been reported to improve the amplification efficiency and specificity of the detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* in soybean seeds (Zhang *et al.*, 1999). The addition of bovine lacto transfer technique optimizer (BLOTTO) in PCR reaction buffers, which comprises skimmed milk powder, phosphate-buffered saline and sodium azide, can also attenuate inhibitory effects of polyphenolic and other compounds derived from plant tissues (De Boer *et al.*, 1995), although to date this has not been tested in amplification reactions with DNA extracted from seeds. However, while these reagents have proven to be effective for the elimination of the effects of PCR inhibitors, their usefulness and their optimal concentrations have to be tested in each case, as some of these reagents might themselves inhibit PCR amplifications when they are used at concentrations that are too high (Wilson, 1997; Koonjul *et al.*, 1999).

PCR has numerous advantages, but the presence of low levels of the target fungus on the seeds can be a problem and may result in a false negative. The major disadvantage of this technique is that it does not provide accurate information concerning the percentage of contaminated seeds, which is an important parameter for seed growers and seed trading companies.

BIO-PCR

Seeds are often infested with fungi at very low levels, and consequently the DNA of the pathogen is not sufficient for the subsequent reactions (De Boer *et al.*, 1995). To overcome this problem, Schaad *et al.* (1995) developed a highly sensitive PCR technique, named BIO-PCR. This consists of a pre-assay incubation step to increase the biomass of the fungal pathogen on the seeds, which is then followed by DNA extraction and amplification by PCR. Initially, this technique was applied mainly for phytopathogenic bacteria, as these are easily and rapidly cultured over 2–3 days in a growth medium (Weller *et al.*, 2000); later, this was also shown to be effective for fungi (Munkvold, 2009).

Several incubation methods have been tested to facilitate rapid and consistent fungal growth, one of which includes the incubation of seeds in liquid fungal growth medium (Phan *et al.*, 2002). However, the fungal growth may be limited or suppressed by the presence of bacteria and the addition of antibiotics to the substrate might not always suppress bacterial growth, which thus decreases the reliability of the PCR assay (Pryor & Gilbertson, 2001; Mbofung & Pryor, 2010). The use of specific media can avoid this problem, increasing the biomass of fungal pathogens, as seen for *L. maculans* and *A. rabiei* from rapeseeds (Taylor, 1993) and chickpea seeds (Phan *et al.*, 2002), respectively.

Other pre-assay methods include an incubation on moistened filter paper, or just on the surface of a plastic Petri dish under conditions of high humidity, such as used to increase the low levels of target *F. oxysporum* f. sp. *lactucae* on lettuce seeds (Mbofung & Pryor, 2010) and *A. radicina* on carrot seeds (Pryor & Gilbertson, 2001). The absence of nutrients allows the development of fungal growth on the seed surface, with little or no bacterial growth (Pryor & Gilbertson, 2001). Also, it is important to determine the incubation period precisely (Schaad & Frederick, 2002), in order to identify a compromise between growth of the target microorganism and limitation of other saprophytic fungi (Phan *et al.*, 2002).

BIO-PCR has several advantages over traditional PCR: increased sensitivity, elimination of PCR inhibitors, and detection of viable cells only, thus avoiding false positives due to detection of DNA from dead cells (Marcinkowska, 2002). The negative aspects of BIO-PCR are that it is more expensive than conventional PCR, especially if selective media are used (Schena *et al.*, 2004), and that it usually requires from 5 to 7 days for the fungal growth, which significantly increases the time required for completion of the assays (Walcott, 2003).

Nested PCR

Another way to obviate the low levels of the target fungus on seeds is the use of nested PCR. This procedure can improve the sensitivity and specificity of the assay, thus allowing detection of a target DNA at several-fold lower levels than for conventional PCR (Chiocchetti *et al.*, 2001). Indeed, nested PCR has been used to detect DNA levels of 10 fg for *Colletotrichum lindemuthianum* in bean seeds (Chen *et al.*, 2007), and of 1 fg for *F. oxysporum* f. sp. *lactucae* in lettuce seeds (Mbofung & Pryor, 2010). However, this molecular assay is more labour intensive, more costly, and more prone to contamination than conventional PCR (McCartney *et al.*, 2003; Atkins & Clark, 2004; Tomlinson *et al.*, 2005).

Real-time PCR

Although conventional PCR is relatively sensitive and specific as well as being rapid, the main disadvantage is that it only gives qualitative data. Real-time PCR is able to generate a specific fluorescent signal detected by an integrated fluorometer to provide real-time analysis of reaction kinetics and so allows quantification of specific DNA targets (Atkins & Clark, 2004; Schena *et al.*, 2004; Ward *et al.*, 2004).

With limited sample manipulation, real-time PCR significantly reduces the risk of false positives due to cross-contamination of the reaction mixtures (Cullen *et al.*, 2001; Gachon *et al.*, 2004; Tomlinson *et al.*, 2005). This technique is less time-consuming than other assays (Guillemette *et al.*, 2004; Chilvers *et al.*, 2007), and is characterized by a high sensitivity in the order of magnitude of a few femtograms of DNA (Chen *et al.*, 2013).

When applied to infected seeds, quantitative real-time PCR assays, with the use of SYBR Green dye and TaqMan-labelled probes, have shown high sensitivity for the detection and quantification of seedborne pathogens. For example, quantitative real-time PCR has been used to detect and quantify *Verticillium dahliae* on spinach seeds (Duressa *et al.*, 2012), *A. brassicae* on cruciferous seeds (Guillemette *et al.*, 2004), *Botrytis* spp. on onion seeds (Chilvers *et al.*, 2007), and *C. lindemuthianum* on dry bean seeds (Chen *et al.*, 2013). Multiplex real-time PCR uses multiple primers (together with probes in the TaqMan assay) in the same reaction, to reduce costs and labour. To ensure adequate specificity and sensitivity, and comparable amplification efficiency of different pathogens in real-time PCR assays, it is critical to choose the appropriate target DNA fragments for the design of the primers and probes.

Magnetic-capture hybridization PCR

Magnetic-capture hybridization (MCH)-PCR combines initial DNA extraction with a purification step that

includes hybridization with a single-stranded DNA probe on magnetic beads, with the subsequent PCR amplification of the relevant DNA region. This technology can capture specific target DNA, thus facilitating detection of specific DNA sequences by PCR (Jacobsen, 1995; Reeves, 1998). MCH-PCR has been successfully used to detect pathogenic microorganisms in materials that contain PCR inhibitory compounds, e.g. *Botrytis aclada* in onion seeds (Walcott *et al*., 2004). Also, a MCH multiplex real-time PCR assay has been developed to detect two different pathogens, *Didymella bryoniae* and *Acidovorax avenae* subsp. *citrulli*, from watermelon and melon seeds (Ha *et al*., 2009). However, the adoption of MCH-PCR is still limited, although, in addition to the above-mentioned advantages of this application, its improved detection of pathogens will facilitate future research.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) of DNA is a recent technology that was developed by Notomi *et al*. (2000) as a simple, cost-effective and rapid method for specific detection of genomic DNA. LAMP uses a set of four or six primers and a thermophilic DNA polymerase from *Geobacillus stearothermophilus* that has strand displacement activity to amplify DNA with high specificity and in less than 1 h (Mumford *et al*., 2006). This technology has several positive features: all of the reactions can be carried out under isothermal conditions; it does not require expensive equipment; and there are fewer preparation steps compared to conventional PCR and real-time PCR assays. Furthermore, as well as being highly specific, the amplification efficiency of LAMP is extremely high, which provides improved sensitivity, and robustness to the inhibitors that usually adversely affect PCR methods (Fu *et al*., 2011). LAMP products can be visualized by gel electrophoresis, by the use of magnesium pyrophosphate, which promotes precipitation of amplified DNA (Fukuta *et al*., 2003; Nie, 2005), with a real-time turbidity reader (Fukuta *et al*., 2004; Mori *et al*., 2004; Thai *et al*., 2004), or with the addition of an intercalating dye, such as SYBR Green I, which produces a colour change if the LAMP reaction is positive (Iwamoto *et al*., 2003; Mumford *et al*., 2006).

To date, the LAMP method has been applied in various assays for the diagnosis of bacterial and viral infections of humans and animals and the detection of plant pathogenic bacteria and viruses in host tissues and in insect vectors (Fukuta *et al*., 2003, 2004; Nie, 2005; Okuda *et al*., 2005). The first applications of LAMP for fungi were reported only recently. LAMP was very effective for the detection of *Fusarium graminearum* in total genomic DNA isolated from ground wheat grains (Niessen & Vogel, 2010) and from contaminated and germinated wheat seeds (Abd-Elsalam *et al*., 2011), thus demonstrating its use for early detection of toxigenic *Fusarium* species in cereals. Over the last 10 years, the LAMP technique has been widely applied in nucleic acid analysis. Therefore, given the numerous advantages provided by LAMP, its application can be extended to a larger number of types of plant tissues, including seeds.

Next-generation sequencing (NGS)

After a first application in basic biological research, NGS technologies have been extended to other fields of application, which have included plant disease diagnosis. Methods based on NGS technologies have been reported for the identification and characterization, in particular, of viruses that affect plants, and less so for pathogenic bacteria and fungi, where a powerful and generic front-line screen has been described (Adams *et al*., 2009, 2013; Kreuze *et al*., 2009; Rwahnih *et al*., 2009; Cantu *et al*., 2011). The application of Life Sciences 454 sequencing allowed analysis of RNAs from a grapevine showing Syrah decline symptoms, which revealed a multiple virus infection, including a novel virus (Rwahnih *et al*., 2009). As NGS has been a valuable technique for rapid identification of disease-causing agents from infected plants, it can also be applied to the detection of fungal pathogens in seeds. This technique has been applied to study the mycobiome of wheat seed, using 454 pyrosequencing, allowing the identification of several fungal genera (Nicolaisen *et al*., 2014). In view of this technology's great potential, the major sequencing platforms used for genome and other sequencing applications, 454 sequencing, AB SOLiD technology and Illumina/Solexa sequencing, are described below.

The first NGS technology that was proposed by Roche for the market was 454 sequencing, which bypasses cloning steps by taking advantage of PCR emulsion, a highly efficient *in vitro* DNA amplification method. It is based on colony sequencing and pyrosequencing. The pyrosequencing approach is a sequencing-by-synthesis technique that measures the release of pyrophosphate by producing light, due to the cleavage of oxyluciferin by luciferase. Currently, the 454 platform can produce 80–120 Mb of sequence in 200- to 300-bp reads in a 4 h run (Morozova & Marra, 2008; Barba *et al*., 2014).

AB/SOLiD technology is sequencing by oligonucleotide ligation and detection (SOLiD). It depends on ligation-based chemistry with di-base labelled probes and uses minimal starting material. Sequences are obtained by measuring serial ligation of an oligonucleotide to the sequencing primer by a DNA ligase enzyme. Each SOLiD run requires 5 days and generates 3–4 Gb of sequence data with an average read length of 25–35 bp (Mardis, 2008; Morozova & Marra, 2008).

Illumina/Solexa sequencing is similar to the Sanger-based methods, because it uses terminator nucleotides incorporated by a DNA polymerase. However, Solexa terminators are reversible, allowing continuation of polymerization after fluorophore detection and deactivation. Sheared DNA fragments are immobilized on a solid surface (flow-cell channel), and solid-phase amplification is performed. At the end of the sequencing run (4 days), the sequence of each cluster is computed and subjected

to quality filtering to eliminate low quality reads. A typical run yields about 40–50 Mb (typical read length of 50–300 bp; Varshney *et al.*, 2009; El-Metwally *et al.*, 2014).

The availability of these NGS assays means that they should now be used to examine the presence of pathogens on or in seeds, especially 454 sequencing that has already been proven to identify fungi on seeds; they may prove useful in future for routine seed diagnosis.

Other newly developed diagnostic techniques

A recently applied tool that can reveal the presence of pathogenic fungi on seeds is known as the 'biospeckle' laser technique. This technique is based on the optical phenomenon of interference that is generated by a laser light that interacts with the seed coat. Examination of seeds under laser light allows the identification of areas with different activities (Braga *et al.*, 2005; Rabelo *et al.*, 2011). As fungi present on the seeds have biological activity, this method can detect their presence on seeds.

Another tool that can distinguish infected seeds from healthy seeds is a multispectral vision system, e.g. VideometerLab instrument, useful to determine the colour, texture and chemical composition of seed surfaces. The combinations of the features from images captured by visible light wavelengths and near-infrared wavelengths were valuable in the separation of healthy spinach seeds from seeds infected by *Stemphylium botryosum*, *Cladosporium* spp., *Fusarium* spp., *Verticillium* spp. or *A. alternata* (Olesen *et al.*, 2011). A similar approach has already been used in a study on malting barley, to detect *Fusarium* spp. (S. Bodevin, Carlsberg Research Centre, Copenhagen, Denmark, personal communication).

Luminex has developed novel technology that, since 2008, has been applied in plant pathogen diagnosis (Ishii *et al.*, 2008). The Luminex PCR system is based on the hybridization between a biotin-labelled PCR product and an oligonucleotide probe coupled to distinct beads that specifically recognize differences in nucleotide sequences. As laborious and time-consuming procedures are not required, the potential of this technique is high and its application may be useful in the context of seed diagnosis. The Luminex xMAP system represents an alternative to ELISA; this technology is based on the use of antibody-coated paramagnetic microspheres (immunobeads) that are stained internally with fluorochromes. These beads act as microscopic ELISA wells. Initially, these techniques were applied in the clinical setting (De Boer & López, 2012), and they are now used for plant pathology applications; e.g. for multiplex detection of seedborne viruses and bacteria (Peters *et al.*, 2007; Bergervoet *et al.*, 2008). This Luminex xMAP technology has been applied for seed detection of several viral pathogens, including *Lettuce mosaic virus* and *Pepino mosaic virus* (Van der Burg, 2009). To date, this technology has not been tested on fungal pathogens in seeds, but in view of its enormous multiplexing capacity, whereby up to 100 pathogens can be analysed simultaneously in a single run, and its rapid analysis, it is an attractive technology for laboratory-based testing of samples (De Boer & López, 2012).

Diagnosis of Important Pathogens Transmitted by Seeds of Vegetable Crops

In the following subsections, the conventional and molecular diagnostic techniques will be examined for each of the pathosystems given in Table 1.

Daucus carota/Alternaria dauci, Alternaria radicina and *Alternaria carotiincultae*

Alternaria leaf blight and alternaria black rot are the most destructive of the diseases of carrot, and they are caused by *Alternaria dauci* and *A. radicina*, respectively. These diseases have spread to all carrot production areas in the world, and they commonly occur when carrots are cultivated under conditions of moderate temperatures, where the leaves are exposed to prolonged periods of wetness due to rainfall, dew or sprinkler irrigation (Pryor, 2002; Rogers & Stevenson, 2010). *Alternaria carotiincultae* is another species that was identified more recently on wild carrot; it is similar in many respects to *A. radicina*, which causes similar disease symptoms (Simmons, 1995).

The efficiency of methods for the detection of *Alternaria* spp. on carrot seeds is influenced by the location of the pathogens on the seed, and by the presence of saprophytic microorganisms. To suppress or eliminate non-target microorganisms, surface sterilization allows more accurate assessment of the incidence of these pathogens. Rinsing carrot seeds in 0.1% sodium hypochlorite has been reported to increase the recovery of *A. dauci* in a 2,4-D blotter assay, and to reduce seed saprophytes (Strandberg, 2002). However, external decontamination can partially suppress the target fungi, thus reducing its incidence. To overcome these drawbacks, the application of heat treatment methods at 100 °C for 1 h allows the detection of high levels of seed infection without compromising the presence of pathogens, while at the same time reducing the masking effects of the seed microflora (Soteros, 1979).

To allow fungal sporulation, and consequently the identification of some species, the standard deep-freeze blotter method for assaying carrot seeds for *Alternaria* spp. infection is effective (Konstantinova *et al.*, 2002; Bulajic *et al.*, 2009). The identification is based on the different morphological features of the reproductive structures of these species: *A. radicina* forms non-catenulate, multicellular, beakless conidia, while *A. dauci* forms solitary, multicellular conidia with long beaks. The conidial dimensions of *A. radicina* are less than those of *A. dauci* (Konstantinova *et al.*, 2002; Pryor & Gilbertson, 2002; Farrar *et al.*, 2004). The morphological features of *A. carotiincultae* differ from those of *A. radicina*: greater mean conidium length, fewer obovoid and subspherical

Table 1 Features of the different seed-detection assays and their effectiveness in detecting seedborne fungal pathogens of vegetable seeds

Diagnostic method	Time required	Sensitivity	Specificity	Ease of implementation	Examples of pathogens detected[a]
Visual examination	Very high	Low	Low	Mycological skills required	*Phomopsis* spp., *Cercospora kikuchii*, *Peronospora manshurica*/soybean seed; *Cylindrocladium parasiticum*/peanut seed; *Colletotrichum dematium*/chilli seed; *Septoria apii*/celery seed
Seed washing technique	Very high	Low	Low	Mycological skills required	*Peronospora manshurica*/soybean seed
Freeze blotter incubation	Low	Low/moderate	Moderate	Mycological skills required	*Alternaria dauci*, *Alternaria radicina*/carrot seed; *Leptosphaeria maculans*/ Brassicaceae seed
Agar medium incubation	Low	Low/moderate	Moderate	Mycological skills required	*Alternaria dauci*, *Alternaria radicina*, *Alternaria carotiincultae*/carrot seed; *Verticillium dahliae*, *Fusarium* spp./ Cucurbitaceae seed; *Botrytis* spp./onion seed
Serology-based assay	High	Moderate/high	Moderate/high	Ease of interpretation	*Macrophomina phaseolina*/cowpea seed
Conventional PCR	Moderate/high	High	High	Molecular biology skills required, ease of interpretation	*Alternaria brassicae*, *Leptosphaeria maculans*/Brassicaceae seed; *Ascochyta lentis*/lentil seed; *Alternaria radicina*/carrot seed; *Phoma valerianella*/lamb's lettuce seed; *Fusarium oxysporum* f. sp. *basilici*/basil seed
BIO-PCR[b]	Moderate	Very high	High	Molecular biology skills required, ease of interpretation	*Alternaria dauci*, *Alternaria radicina*/carrot seed; *Alternaria brassicae*, *Leptosphaeria maculans*/Brassicaceae seed; *Ascochyta rabiei*/chickpea seed; *Fusarium oxysporum* f. sp. *lactucae*/ lettuce seed
Nested PCR	Moderate	Very high	High	Molecular biology skills required, ease of interpretation	*Colletotrichum lindemuthianum*/bean seeds; *Fusarium oxysporum* f. sp. *lactucae*/lettuce seeds
Real-time PCR	High	Very high	High	Molecular biology skills required	*Alternaria brassicae*, *Plasmodiophora brassicae*/Brassicaceae seed; *Didymella bryoniae*/Cucurbitaceae seed; *Botrytis* spp./onion seed; *Verticillium dahliae*/ spinach seed; *Colletotrichum lindemuthianum*/bean seed; *Fusarium oxysporum* f. sp. *basilici*/basil seed
MCH-PCR[c]	High	Very high	High	Molecular biology skills required	*Didymella bryoniae*/Cucurbitaceae seed; *Botrytis* spp./onion seed
Laser biospeckle technique	High	High	High	Technological skills required	*Fusarium oxysporum, Aspergillus flavus*, *Sclerotinia* spp./bean seed
Videometer	High	High	High	Technological skills required	*Stemphylium botryosum*, *Cladosporium* spp., *Fusarium* spp., *Verticillium* spp., *Alternaria alternata*/spinach seed

[a]All of these pathogens are referenced in the text.

[b]BIO-PCR, with pre-PCR incubation step to increase biomass of the fungal pathogen.

[c]MCH-PCR, magnetic-capture hybridization PCR.

conidia, and greater frequency of conidia that are produced in chains of two, or less commonly, three (Pryor & Gilbertson, 2002).

The production of conidia by many *Alternaria* spp. is subject to the influence of a number of environmental conditions, such as temperature and quantity and quality of light. Thus, this can affect the sensitivity and reproducibility of seed assays performed on blotter paper. The identification of *Alternaria* spp. based on its vegetative growth on selective media is subject to fewer variations than methods based on the production and identification of the conidia, such as the freeze blotter method (Pryor *et al*., 1994; Lopes & Martins, 2008). For the detection of *A. dauci* on infested carrot seeds, a modified agar allows high levels of seed infection to be obtained, thus providing a more sensitive assay than the blotter method.

Alternaria radicina can be identified through the use of selective media created specifically for this species (Pryor

et al., 1994) or the addition of selective fungicides and antibiotics to the medium (Strandberg, 2002). These suppress the growth of seed saprophytes but not of the target pathogen, thus eliminating the additional step of pretreating the seeds before the assay.

Selective media based upon carrot leaf extracts combined with fungicides and/or bactericides have been designed for both *A. radicina* and *A. dauci*, although these are particularly sensitive for the detection of *A. dauci* on infested carrot seeds, through the promotion of profuse sporulation (Strandberg, 2002). *Alternaria dauci* colonies are brown or dark brown with olive-grey aerial mycelia, and in comparison to the other *Alternaria* spp., *A. dauci* colonies can be recognized by the production of a brown diffusible pigment in the medium (Lopes & Martins, 2008). *Alternaria radicina* isolates grow slowly with irregular colony margins and produce dendritic crystals and a diffusible yellow pigment (Pryor & Gilbertson, 2002).

Although *A. radicina* and *A. carotiincultae* are very similar species, they can be differentiated on the basis of their culture characteristics on acidified potato dextrose agar. Unlike colonies of *A. radicina*, those of *A. carotiincultae* grow more rapidly and do not produce crystals or pigment. These different characteristics might be associated with the production of radicinin (which is significantly higher in *A. radicina* isolates than *A. carotiincultae* isolates), thus providing a useful diagnostic characteristic (Park *et al.*, 2008).

In BIO-PCR, an incubation step of the seeds can increase the fungal biomass. Then, the use of specific primers for *A. dauci* and *A. radicina* during the PCR assays is highly sensitive, with detection of even low infection levels, and differentiation between these *Alternaria* spp. (Konstantinova *et al.*, 2002). During the DNA extraction, the use of a silica matrix allows higher yields of DNA to be obtained. The target *A. radicina* DNA sequence is consistently amplified if the DNA extracts from infested seeds are diluted with Tris-EDTA (TE) buffer, or with the addition of skimmed milk to the PCR mixture (Pryor & Gilbertson, 2001). Data obtained using the blotter method and incubations on selective media are similar to those obtained after the application of molecular methods, although the molecular methods are less time-consuming.

Brassica spp./*Alternaria brassicae*, *Leptosphaeria maculans* and *Plasmodiophora brassicae*

Alternaria brassicae and *L. maculans* are the causal agents of black spot disease and blackleg disease, respectively. These are important seedborne pathogens that affect *Brassica* spp., and can cause serious reductions in crop yields (Howlett *et al.*, 2001; Lancaster, 2006). Another economically important disease of the Brassicaceae family is clubroot, which is caused by the obligate parasite *P. brassicae*, a soilborne, and also seed-transmissible, pathogen (Dixon, 2009; Hwang *et al.*, 2012).

For the identification of *A. brassicae*, PCR and real-time PCR assays have been applied, using two specific primer pairs that were designed on the basis of the sequences of two clustered genes that are potentially involved in pathogenicity. Before the DNA extraction from seeds, an incubation step of a few days is advisable to increase the fungal biomass, using 2% malt extract, 2% dextrose and 0.1% peptone liquid culture medium. The mycelia and conidia can then be separated from the seeds and used for DNA extraction (Guillemette *et al.*, 2004).

To detect *L. maculans* in seed samples, the filter paper/freeze method can be used, although PCR with a species-specific primer pair is equally effective and less time-consuming (Mahuku *et al.*, 1996; Chen *et al.*, 2010). BIO-PCR with a specific medium for *L. maculans* can help to increase the biomass of this fungus on Brassicaceae seeds (Taylor, 1993).

A real-time PCR-based protocol that uses specific primers has been developed for the quantification of *P. brassicae* resting spores that occur as external contaminants on canola seeds. Quantitative PCR analysis can be performed on undiluted DNA extracted from the seeds, or after dilution to avoid problems due to PCR inhibitors (Rennie *et al.*, 2011).

Cucurbitaceae/*Didymella bryoniae*, *Verticillium dahliae* and *Fusarium* spp.

Gummy stem blight (black rot) is a serious foliar disease that affects Cucurbitaceae crops. The disease is caused by the fungus *D. bryoniae*, which produces lesions on the stems and leaves. *Didymella bryoniae* can also be spread by seeds because it is located both externally on and internally in the seeds (Sudisha *et al.*, 2006). *Verticillium dahliae* and *Fusarium* spp. are destructive soilborne and seedborne pathogens that can infect many economically important agricultural crops worldwide, including Cucurbitaceae (Trionfetti Nisini *et al.*, 2002; Rampersad, 2008).

PCR-ELISA has been used successfully to detect *D. bryoniae* on crude extracts of fungal samples isolated from infected cucurbit plants. Although less sensitive than gel electrophoresis, PCR-ELISA is a highly specific, simple and rapid assay, and it can also be used to identify *D. bryoniae* in cucurbit seed lots (Somai *et al.*, 2002). To obtain greater sensitivity, specific real-time PCR can be used (Ling *et al.*, 2010), and if other pathogens are present in addition to *D. bryoniae*, MCH-multiplex real-time PCR can be applied to identify all of the microorganisms in these seeds. This last technique is 10-fold more sensitive than direct real-time PCR (Ha *et al.*, 2009).

Verticillium dahliae infections on seeds affect the seed coat, cotyledons and radicle (Maruthachalam *et al.*, 2013). For its isolation from pumpkin seeds, a semiselective medium can be used (Termorshuizen *et al.*, 1998), and then the colonies obtained can be subcultured onto potato dextrose agar medium for species identification (Rampersad, 2010). Direct incubation of dissected watermelon seeds (i.e. without the testa) on Komada's selective medium for *Fusarium* spp., and incubation of entire

seeds on the same medium or on 2% agar medium, allows both external and internal *Fusarium* spp. to be detected. The incubation of seeds without the testa allows the development of the endogenous *Fusarium* spp. in the seeds (Boughalleb & El Mahjoub, 2006).

Recently Cohen *et al.* (2014) found another pathogen on cucurbits, *Pseudoperonospora cubensis*, which might be seedborne and seed-transmitted. Species-specific PCR assays have shown that *P. cubensis* occurs in the ovaries, fruit seed cavity, and seed embryos of cucurbits.

Allium cepa/Botrytis spp.

Seven *Botrytis* spp. have been associated with diseases of *Allium* crops: *B. aclada*, *B. allii*, *B. byssoidea*, *B. cinerea*, *B. porri*, *B. squamosal* and *B. tulipae* (Mohan & Schwartz, 2005). *Botrytis aclada*, *B. allii*, *B. byssoidea* and *B. porri* are considered the primary causal agents of neck rot of onion, a disease that can develop during storage and that can cause severe loss of onion bulbs. Although *B. squamosa* and *B. tulipae* have also been associated with neck rot, these species are not typically the primary causes (Chilvers *et al.*, 2004; Chilvers & Du Toit, 2006).

For the determination of the health of onion seeds, various procedures can be used to isolate *Botrytis* spp. The seeds can be incubated on agar medium and, to prevent the onset of fast-growing fungi that can outgrow *Botrytis* spp. on or in the seeds, they can be plated on a selective medium such as Kritzman's agar. This provides several advantages over other media, such as freedom from secondary contaminants and ease of identification of *Botrytis* spp. (Kritzman & Netzer, 1978). To detect propagules on seed surfaces, seeds can be rinsed under running deionized water for 1 h to remove the spores of fast-growing contaminating fungi. For an internal seed assay, surface sterilization using a disinfectant such as NaOCl is necessary before plating (Du Toit *et al.*, 2004; Chilvers & Du Toit, 2006). Colonies of *Botrytis* spp. obtained after seed incubations can be transferred to acidified potato dextrose agar for species determination (Du Toit *et al.*, 2002).

For rapid and sensitive detection of *B. aclada* in onion seeds, a MCH-PCR assay can be applied (Walcott *et al.*, 2004). Real-time PCR using SYBR Green chemistry and primer pairs specific for *B. aclada*, *B. allii* and *B. byssoidea* can be used to quantify *Botrytis* spp. on onion seeds, with the detection of as little as 10 fg genomic DNA extracted from pure cultures of *B. aclada* and *B. allii* (Chilvers *et al.*, 2007).

Concluding Remarks

The present review has covered the diagnostic methods that can be used to detect and identify fungal pathogens carried by seeds of vegetable crops. As well as reducing the quantity and quality of the seed harvested, seedborne pathogens can be preserved in seed lots, which can provide a massive boost to the spread of plant pathogens (Mancini & Romanazzi, 2014). Seeds are highly vulnerable to infection and/or contamination, and seed diagnosis can be particularly difficult, because in most cases, seeds do not show visible symptoms, unlike other plant tissues (Schaad *et al.*, 2003).

Conventional seed detection assays that include visual examination and incubation methods have been extensively used, especially in the past, before the development of molecular diagnostic techniques. In recent years, these molecular techniques have increasingly replaced the traditional methods, which have the drawback of being time-consuming, laborious, and not always reliable. Despite this, traditional methods can still be used to ensure detection of particular fungal pathogens, with the advantage that they are cheaper than the molecular alternatives. For example, to detect pathogenic species of *Alternaria* on infected carrot seeds, traditional methods are still widely applied because these species show distinctive morphological features that allow them to be distinguished from each other. However, in many cases, accurate identification of pathogenic organisms using these traditional diagnostic assays is difficult, and these methods normally only work well when the seed samples contain high levels of the target pathogens. The application of nucleic acid-based detection methods allows these shortcomings to be overcome, as these are more specific and sensitive, and they provide data that are easy to interpret in a short time. However, PCR inhibitors can limit the applicability of these molecular technologies, although the integration of conventional or real-time PCR with BIO-PCR and MCH-PCR allows this problem to be avoided, and improves the detection of seedborne pathogens.

Seed health testing is a central issue in the context of the phytosanitary regulations set up by national governments and by groups of countries, such as the European Community, as a wide range of pests can be carried in seed lots. Sampling in seed health testing is a key factor (Morrison, 1999) and international organizations, such as the European and Mediterranean Plant Protection Organization (EPPO), the International Seed Health Initiative (ISHI), the International Seed Testing Association (ISTA) and the International Society for Plant Pathology (ISPP), have the task of developing and approving standard methods for seed health testing and appropriate seed sampling procedures, so that each test sample is as homogeneous as possible and representative of each lot. To improve seed quality control, novel and efficient methodologies, such as NGS and non-destructive testing methods, are increasingly emerging. Although these methods are not yet widely used for seed diagnosis, due to their great potential, they will probably be increasingly applied for the detection of pathogens on seeds.

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