Diagnosis and Detection of Seed-Borne Fungal Phytopathogens

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Abstract

Food losses due to crop infections caused by different pathogens such as bacteria, viruses and fungi are persistent issues in agriculture for centuries across the globe. The timely detection and appropriate identification of casual agents associated with diseases of crop plants or seeds are considered to be the most important issue in formulating the management strategies. Seed health testing to detect seed-borne pathogens is an important step in the management of crop diseases. Specificity, sensitivity, speed, simplicity, cost-effectiveness and reliability are the main requirements for the selection of seed health test methods. 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, specificity and accuracy. Polymerase chain reaction (PCR) has emerged as a tool for the detection of microorganisms from diverse environments. Thus far, it is clear that nucleic acid-based detection protocols exhibit higher level of sensitivity than conventional methods. Unfortunately, PCR-based seed tests require the extraction of PCR-quality DNA from target pathogens in backgrounds of saprophytic organisms

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and inhibitory seed-derived compounds. The inability to efficiently extract PCR-quality DNA from seeds has restricted the acceptance and application of PCR for the detection of seed-borne pathogens. To overcome these limitations, several modified PCR protocols have been developed including selective target colony enrichment followed by PCR (Bio-PCR). These techniques seek to selectively concentrate or increase target organism populations to enhance detection and have been successfully applied for detecting fungi in seed. Ultimately, improved protocols based upon PCR, ELISA, etc. will be available for the detection of all seed-borne pathogens and may supersede conventional detection methods. This chapter provides a comprehensive overview of conventional and modern tools used for the early detection and identification of seed-borne fungal pathogens.

5.1 Introduction

Seed-borne pathogens possess a serious threat to seedling establishment. Close association of the pathogens with seeds facilitates their long-term survival, introduction into new areas and widespread dissemination. Under such conditions, elimination is the most effective disease management strategy accomplished by using seed detection assay to screen and reject infested seed lots before sowing/planting or its distribution to the farmers or seed growers. Transboundary spread of pathogens is a major concern today. Precise detection methods are essential for seed-borne pathogens to support seed health strategies. While choosing a method, it is essential to see that it is reliable, less time-consuming, cost-effective, reproducible and sensitive. The conventional seed health testing methods are being used in the identification of fungus up to species level. The considerable advancement in molecular biology has facilitated rapid identification/detection of seed-borne pathogens. Over 100 years of seed health studies, many new methods were developed, or older methods were modified, but all of them used for the detection and identification of seed-borne organisms have to fulfil six main requirements (Ball and Reeves 1991):

(i) ***Specificity*** – the ability to distinguish a particular target organism from others occurring on tested seeds.
(ii) ***Sensitivity*** – the ability to detect organisms at low incidence in seed stocks.
(iii) ***Speed*** – less time requirements, to enable prompt action against the target pathogen(s).
(iv) ***Simplicity*** – minimization of a number of examination stages to reduce error and enable testing by a staff not necessarily highly qualified.
(v) ***Cost-effectiveness*** – costs should determine acceptance to the test.
(vi) ***Reliability*** – methods must be sufficiently robust to provide repeatable results within and between samples of the same stock regardless who performs the test.

5.2 Why Detection of Seed-Borne Fungal Pathogens is Important?

- Seed-borne fungal pathogens present a serious threat to seedling establishment and hence may contribute as potential factor in crop failure.
- Seeds not only facilitate the long-term survival of these pathogens but also may act as a vehicle for their introduction into newer areas and their widespread dissemination.
- Seed-borne fungal pathogens are able to cause catastrophic losses to food crops and hence directly linked to the food security.
- Unlike infected vegetative plant tissues, infested seeds can be asymptomatic, making visual detection impossible.
- Additionally, fungal pathogen's populations on seeds may be low, and the infested seeds may be non-uniformly distributed within a lot.

5.3 Detection Methods for Seed-Borne Fungal Pathogens

The following methods are used to detect seed-borne fungal pathogens which include conventional and modern methods.

5.3.1 Conventional Detection Methods

5.3.1.1 Visual Examination of Dry Seeds

The first step of the detection of seed-borne pathogens is examination of dry seeds with unaided eye (naked eye) or with the magnifying glasses (hand lens). In certain cases, infected seeds exhibit different characteristic symptoms produced by various seed-borne fungal pathogens on seed surface, viz. seed rot, seed necrosis, shrunken seed, seed discolouration, shrivelling, etc. (Table 5.1 and Fig. 5.1). Besides these symptoms, dry seeds are examined for the presence of admixtures such as sclerotia, fungal fructification such as pycnidia and acervuli, smut balls and smut sori, etc. In this method stereoscopic microscope, hand lens or naked eye can be used for a sample consisting of 400 or more seeds. By this examination some additional significant risks can also be eliminated, e.g. weed seed contaminants, insect pests and abnormal seeds. Seed may be soaked in water or other liquids to make pathogen structures, e.g. pycnia, and symptoms, i.e. anthracnose, on the seed coat more visible.

Visual examination method may be coupled with automatic devices that sort seeds based on visuals of physical characteristics (Paulsen 1990; Walcott et al. 1998) to reduce seed lot infestation. But, as a limitation, these systems usually have low detection sensitivity, which makes these devices less useful in decision-making system for rejection of seed lots. 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.

Table 5.1 Visual sign/symptoms of some major seed-borne fungi on various crop seed

S. no.	Crop	Visual sign or symptom on seed	Possible fungi associated	References
1.	Barley	Scald symptoms	*Rhynchosporium secalis*	Lee et al. (1999)
2.	Carrot	Seed rot	*Alternaria radicina*	Gaur (2011)
3.	Celery	Pycnidia embedded in the seed coat	*Septoria apii*	Horst (2008)
4.	Cereals	Normal seed is replaced by sori of spores	Smut, bunt or ergot in cereals	Warham et al. (1996)
5.	Chick pea	Small and wrinkled seed	*Fusarium oxysporum* f. sp. *ciceri*	Khare (1996)
		Ashy brown discolouration in seeds	*Ascochyta rabiei*	Khare (1996)
		Blackish seed coat	*Alternaria alternata*	Khare (1996)
		Reduction in seed size	*Ascochyta rabiei*	Gaur (2011)
6.	Chilli	Acervuli and microsclerotia	*Colletotrichum dematium*	Kumar et al. (2004)
7.	Coriander	Hypertrophied seed	*Protomyces macrosporus*	Khare (1996)
8.	Crucifers	Reduction in seed size and seed rot	*Phoma lingam*	Gaur (2011)
		Shrivelling	*Alternaria brassicae*, *A. raphani* and *A. alternata*	Rude et al. (1999)
9.	*Dolichos lablab*	Red discolouration around micropyle (red nose)	*Stemphylium botryosum*	Gaur (2011)
		Brown spot	*Colletotrichum lindemuthianum*	Gaur (2011)
10.	Lentil	Ashy brown discolouration in seeds	*Ascochyta fabae* f. sp. *lentis*	Khare (1996)
11.	Maize	White streaks with black spore masses near the tips	*Nigrospora* sp.	Agarwal and Sinclair (1997)
		Seeds exhibit white streaks	*Fusarium moniliforme*	Khare (1996)
		Seed rot	*Fusarium graminearum*	Gaur (2011)
12.	Onion	Seed rot	*Alternaria porri*	Gaur (2011)
		Shrunken seeds	*Peronospora destructor*	Gaur (2011)
13.	Pea	Brown spot	*Ascochyta pisi*	Gaur (2011)
		Seed rot	*Mycosphaerella pinodes*	Gaur (2011)
14.	Peanut	Speckles	*Cylindrocladium parasiticum*	Randall-Schadel et al. (2001)
15.	Rice	Light pink discolouration	*Fusarium graminearum*	Sachan and Agarwal (1995)
		Ash grey discolouration	*Alternaria alternata*	Sachan and Agarwal (1995)
		Black discolouration, dark brown spots and light to dark brown dot-like spots	*Helminthosporium oryzae* [*Cochliobolus miyabeanus*]	Sachan and Agarwal (1995)
		Light brown discolouration	*Sarocladium oryzae*	Sachan and Agarwal (1995)

(continued)

Table 5.1 (continued)

S. no.	Crop	Visual sign or symptom on seed	Possible fungi associated	References
16.	Sesame	Hyphae and sclerotia on seed coat	*Macrophomina phaseolina*	Khare (1996)
17.	Sorghum	Completely deformed	*Acremonium* sp.	Agarwal and Sinclair (1997)
		Shrunken seeds	*Sclerospora sorghi*[a]	Gaur (2011)
18.	Soybean	Purple stain	*Cercospora kikuchii*	Murakishi (1951) and Khare (1996)
		Fine cracks and mould, starting near the hilum	*Phomopsis longicolla*	Li (2011)
19.	Wheat	Bunted seed	*Tilletia tritici*	Warham (1986)
		Shrivelled rough scabby appearance	*Fusarium* sp.	Warham et al. (1996)
		Black point on seed	*Alternaria alternata*	Khare (1996)

[a]Not seed-borne but affect seed

Fig. 5.1 Visual symptoms caused by fungal pathogens on seeds after harvesting (**a**–**d**) and in standing crops (**e**–**h**): Karnal bunt of wheat (**a**), false smut of paddy (**b**), kernel smut/bunt of paddy (**c**–**d**), loose smut of wheat (**e**), ergot of pearl millet (**f**), green ear disease of pearl millet (**g**), smut disease of pearl millet (**h**)

5.3.1.2 Microscopic Examinations

(a) **Examination of Seed Washings**

This method is used to detect seed-borne pathogens which are loosely present on the seed surface. This method is mostly used for the detection of fungi causing smuts, bunts, downy mildew, powdery mildew and rust with the important exception of loose smut of wheat and barley which are internally seed-borne diseases. For seed washing test, seed samples (50 seeds) are placed in test tubes containing sterile distilled water (10 ml) and a few drops (10–20) of 95% ethyl alcohol or a detergent. The sample tubes are agitated in a mechanical shaker for 10 min. The aqueous

suspension is then centrifuged at 1000 rpm for 10 min. The supernatant is poured off and the pellet is re-suspended in 2 ml of sterile water. Spores or fungal structure present in the suspension can be viewed by examining a few drops of the suspension under the light microscope.

(b) **NaOH Seed Soak Method**

The NaOH seed soak method was first used by Agarwal and Srivastava (1981). This method is applied for the detection of Karnal bunt of wheat and bunt (kernel smut) of paddy. In this method seeds are soaked in 0.2–0.3% NaOH solution for 24 h at 25–30 °C. Next day the solution is decanted and the seeds are thoroughly washed in tap water. After washing, the seeds are spread over blotter paper so that the excess moisture is absorbed by blotter. Now the seeds are examined visually. The wheat seeds showing black to shiny black discolouration may contain Karnal bunt infection of *Tilletia indica.* This may be confirmed by rupturing suspected seed with a fine needle in a drop of water, the bunt spores (teliospores) will be released, if the suspected seed is infected. Similarly, the infection in paddy seeds due to bunt or kernel smut disease of paddy caused by *Tilletia barclayana* can also be detected. Likewise, by treating the rice seeds with NaOH (0.2%), the infection by *Trichoconiella padwickii* could be inferred by the change of colour of the diseased portion of infected seeds to black (Singh and Maheshwari 2001).

(c) **Whole Embryo Count Method**

This method is used when seed-borne infection is deep seated in the seed tissues such as embryo in case of loose smut of wheat and barley. The embryo count method was first used by Skvortzov (1937) for detecting loose smut pathogen *Ustilago nuda* var. *tritici*. He dissected the embryos, macerated them with NaOH and then stained them with aniline blue. This method is completed in 3–4 days. This method was modified by Agarwal et al. (1978) as follows (Fig. 5.2):

5.3.1.3 Incubation Methods

(a) **Testing on Agar Media:** In agar tests seeds are incubated on agar media for a particular length of time and optimum temperature under alternating light and dark cycles. The associated fungi are detected based on their morphological and habit characters on seed surface and colony characters on the medium. It is used to detect *Alternaria*, *Bipolaris*, *Curvularia*, *Fusarium*, etc. in infected seeds.

(b) **Blotter Testing:** Doyer (1938) and de Temp (1953) were first to adopt blotter paper method in seed health management. This test is used to detect infection of seeds, and in certain cases, infection of the germinated seedlings can also be detected by this method. Blotter method is the most widely used seed health assay. Mainly this method is of two types:

(i) **Standard Moist Blotter (SMB) Method**

In the standard blotter test, seeds are sown in Petri dishes containing 1–3 layers of water or buffer-soaked absorbing (blotting) paper or cellulose pads

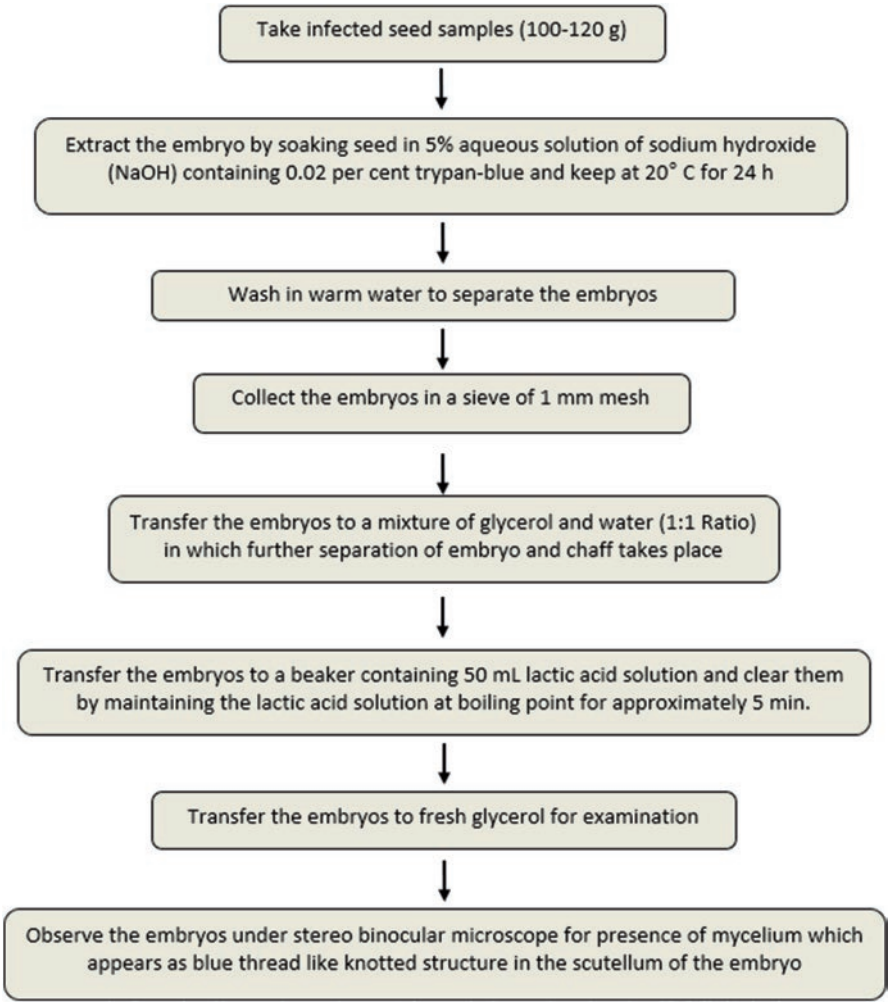

Fig. 5.2 General protocol for whole embryo count method for detection of loose smut of wheat

for a couple of days depending on the fungus and type of seed tested (Marcinkowska 2002). In general, 10–20 non-sterilized seeds (depending on the seed size) are placed equidistant from each other in Petri dish and incubated at 25 ± 2 °C with alternate cycles of 12 h of light and 12 h of darkness for 7–10 days. In the blotter test, seeds are subjected to conditions that enable pathogen growth and expression during the incubation period (Fig. 5.3). After the incubation period, the seeds are examined under a stereomicroscope for the presence of fungal colonies, and their characteristics are recorded for the identification of the fungal pathogens.

The seed must be surface-sterilized prior to its placement on blotter paper in Petri dish, if the internally seed-borne fungal pathogens are to be

Fig. 5.3 Incidence of seed mycoflora on pea seeds under blotter testing method after 10 days of incubation

detected. Seeds may be immersed in a NaOCl solution containing 1% chlorine for 10 min or in 1.7% NaOCl solution for 1 min followed by immersion in 70% chlorine for 10 min (ISTA 1966). The germination of seeds may be obstructed by wetting the blotting paper with 0.1–0.2% 2,4-D. This procedure has been used for the detection of *Leptosphaeria maculans* (anamorph *Phoma lingam*) in crucifer seeds (Hewett 1977) and for routine seed health testing of common bean and soybean (Dhingra et al. 1978).

(ii) **Deep Freezing Blotter (DFB) Method**

The DFB method is used to detect a wide range of fungi which are able to grow easily from seeds in the presence of humidity. After plating seeds as described in the SMB method, the Petri dishes are incubated at 20 ± 2 °C for 24 h and then transferred to a − 20 °C freezer for 24 h followed by incubation at 20 ± 2 °C for 5 days under cool white fluorescent light with alternating cycles of 12 h light and 12 h darkness. Pure cultures are obtained through hyphal-tip and single-spore isolation techniques and maintained on carrot potato agar (CPA) slants for further studies. Fungi are identified using cultural, biochemical, macromorphological and micromorphological characteristics as described by Raper and Fennel (1965), Booth (1971), Ellis (1971) and Domsch et al. (1980).

The percentage of seed infection in each sample and the percentage of infection in each region are determined by the following formulae:

$$\text{Mean rate of seed infection} = \frac{\text{Number of seeds on which a fungal species identified}}{\text{Number of seeds tested}} \times 100$$

$$\text{Mean of regional infection} = \frac{\text{Frequency of sample on which a fungus identified}}{\text{Number of samples collected}} \times 100$$

For species-level identification, the fungi are isolated on potato dextrose agar (PDA) and maintained at 24 ± 1 °C for 7–10 days. The identification is conducted using colony colour, colony texture pattern, arrangement of spore on the conidiophores, spore shape and size (Watanabe 2002; Leslie and Summerell 2006; Utobo et al. 2011).

The blotter method has been coupled with scanning electron microscopy (SEM) for the detection of seed-borne fungi (Alves and Pozza 2009). The seeds of common bean (*Phaseolus vulgaris* L.), maize (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) were submitted to the standard blotter test. The specimens were prepared and observed with the standard SEM methodology. It was possible to identify *Fusarium* sp. on maize, *C. gossypii* var. *cephalosporioides* and *Fusarium oxysporum* on cotton and *Aspergillus flavus*, *Penicillium* sp., *Rhizopus* sp. and *Mucor* sp. on common bean (Alves and Pozza 2009).

(c) **Seedlings Symptoms Test and Grow-Out Test**

Seedlings symptoms test is based on the characteristic symptoms produced by seed-borne fungi on growing seedlings under controlled conditions, whereas in grow-out test, plants are grown beyond the seedling stage in near-optimum conditions of temperature and moisture in sterile medium, i.e. sand, and water-agar medium, and the seedlings/plants are observed for symptoms of the fungal pathogens. It can facilitate the detection of a number of fungal pathogens associated with seed rotting and other symptoms at seedling stage, e.g. fungal pathogens causing seedling diseases as *Alternaria*, *Bipolaris*, *Fusarium*, *Pyricularia*, etc. This method involves the planting of a certain number of seeds, preferably on sterile soil for determining the number of infected plants and calculating the percent infected plants out of the total number of seed sown. These test results are helpful in assessing field performance and estimating the number of infection loci/unit area, if the seed lot under investigation is used for cultivation by farmers. Infection of soybean seeds by *Colletotrichum truncatum* was detected by this method (Dhingra et al. 1978). This method is very effective in the case of non-cultivable obligate pathogens causing downy mildew diseases. However, it requires large greenhouse space, and also it is time-consuming, making it unsuitable for testing a large number of seed lots. There are a number of seedling symptoms and grow-out tests as follows:

(i) **Test Tube Agar Method**

This method was developed by Khare, Mathur and Neergaard in 1977. It is used for the detection of *Septoria nodorum* in wheat seeds and is very

useful for assaying the small quantity of high cost material. In this method infection of root can also be examined. Fungal pathogens of cereals like *Drechslera* sp., *Bipolaris* sp. and *Septoria* sp. can be easily detected. Steps used in procedure are as follows:

1. 15 ml water agar is taken in test tube, sterilized and solidified with a slight slant.
2. One seed is sown in each test tube and incubated at 28 ± 1 °C with 12 hours alternating cycles of light and darkness.
3. Seedlings are examined after 14 days for the typical symptoms of disease in the coleoptiles.
4. The symptoms can be easily studied being visible on roots as well as on green parts.

(ii) **Hiltner's Brick Stone Method**
It was developed by Hiltner in 1917. Sterile crushed brick stone with a maximum piece size of 3–4 mm is used to fill in plastic pots up to ¾th of their capacity. The crushed brick stone in the pot is saturated with water and seeds are placed 1 cm deep. The pots are kept in darkness at room temperature, and observations for disease symptoms are recorded after 2 weeks by removing the seedlings. It is a good method for testing field performance giving information on seedling symptoms. It is also used for testing treated seed.

(iii) **Sand Method**
This method is similar to Hiltner's brick stone method except that in place of sterile crushed brick stone, sterilized sand is used.

(iv) **Standard Soil Method**
A pre-sterilized uniform soil mixture containing four parts clay, six parts peat and essential amount of fertilizer is filled in plastic multi-pot trays. After sowing the seed, appropriate moisture should be maintained. The symptoms are observed after incubation for 2–4 weeks depending on the kind of seed and temperature.

(d) **Selective Media**

It is a direct method of seed testing in which seed-borne pathogens are allowed to grow on specific media. The use of selective media for the detection of pathogens is more reliable than blotter or agar method. This can be done by directly plating surface-sterilized seed samples or seed wash liquid onto artificial media, followed by adequate incubation under favourable conditions. Once a fungal pathogen is isolated, it can be identified by its cultural, morphological or biochemical characteristics. Selective artificial media are developed that use antibiotics, fungicides, selected carbon and nitrogen sources and other inhibitory compounds to retard the growth of non-target microflora while allowing the target pathogen to grow. For example,

potato dextrose agar is useful for the detection of *Septoria nodorum* in wheat, while PCNB agar is a selective medium for the detection of *Fusarium* species in cereals. The list of some selective and semi-selective media for different seed fungi is given in Table 5.2.

Table 5.2 General/selective media and temperature requirements that favour the development of seed-borne fungal pathogens

S. no.	Name of seed-borne fungal pathogen	Nutrient medium	Incubation temp. (°C)	References
1.	*Botrytis cinerea*	Selective media: *Botrytis* selective medium (BSM) and *Botrytis* spore trap medium (BSTM)	25 °C	Edwards and Seddon (2001)
2.	*Botrytis cinerea*	Potato dextrose agar (PDA)	20–22 °C	Mirzaei et al. (2008)
3.	*Botryodiplodia theobromae*	PDA	28 °C	Fu et al. (2007)
4.	*Lasiodiplodia theobromae*	Selective medium	25 °C	Cilliers et al. (1994)
5.	*F. oxysporum* f. sp. *niveum*	PDA/lima bean agar	25 °C	Zhang et al. (2005)
6.	*F. oxysporum* f. sp. *cucurbitae*	*Fusarium* selective medium (FSM)	25–37 °C	Mehl and Epstein (2007, 2008)
7.	*F. solani* f. sp. *cucurbitae*	*Fusarium* selective medium (FSM)	22 °C	Mehl and Epstein (2007)
8.	*Trichoconiella padwickii*	Semi-selective media	28–30 °C	Muthaiyan (2009)
9.	*Fusarium graminearum*	Semi-selective media (NSA, SRA-FG)	25–28 °C	Segalin and Reis (2010)
10.	*Fusarium* species	Semi-selective medium (MGA 2.5 + carnation leaves)	25 °C	Thompson et al. (2013)
11.	*Exserohilum turcicum*	Semi-selective medium (DRR-Reis)	25 ± 2 °C	De Rossi and Reis (2014)
12.	*Alternaria brassicicola* (crucifer seeds)	Semi-selective media (CW medium)	24 °C	Wu and Chen (1999)
13.	*Fusarium* species in cereals	Dichloran chloramphenicol peptone agar (DCPA)	25 °C	Andrew and Pitt (1986)
14.	*Phoma betae*	Hold fast method	20 °C	Mangan (1971)
15.	*Fusarium moniliforme*	Modified Czapek's dox agar medium (MC_ZA)	26–28 °C	Agarwal and Singh (1974)
16.	*Pyricularia oryzae*	Guaiacol agar	25 °C	Kulik (1975)
17.	*Stagonospora nodorum*	SNAB (*Stagonospora nodorum* agar for barley)	20 °C	Cunfer and Manandhar (1992)
18.	*Septoria nodorum* [*Stagonospora nodorum*]	Selective media SNAW	20 °C	Manandhar and Cunfer (1991)

(continued)

Table 5.2 (continued)

S. no.	Name of seed-borne fungal pathogen	Nutrient medium	Incubation temp. (°C)	References
19.	*F. graminearum*	Toxoflavin-based selective medium	25 °C	Jung et al. (2013)
20.	*Curvularia lunata*	CS medium with 200 ppm carbendazim +200 ppm streptomycin and CR medium with 200 ppm carbendazim +200 ppm rifampicin	25 °C	Deshpande (1993)
21.	*Rhynchosporium secalis*	Lima bean agar medium	15–20 °C	Lee et al. (1999)
22.	*Fusarium* species in cereals	PCNB agar	22–25 °C	Pastircak (2007) and Alborch et al. (2010)

5.3.2 Serological Detection Techniques

The seed-infecting fungal communities may comprise the saprobes which can grow rapidly over the target fungal pathogens. These fast-growing saprobes arrest their isolation; and examination of their morphological characteristics becomes difficult and confusing. In the case of rice seed-borne pathogens, such situation exists, where about 30 fungal phytopathogens infecting rice have been reported to be seed-borne (Mew et al. 1988). Additionally, the presence of very closely related strains, race or even fungal species on the seeds makes the detection morphologically almost impossible. Therefore, more sensitive techniques such as immunoassay and nucleic acid-based protocols are needed to overcome this issue. Since pure culture of the pathogens is not needed in serological detection protocols, these techniques could be applied to detect biotrophic as well as necrotrophic seed-borne pathogens (Mancini et al. 2016).

After the first use of enzyme-linked immunosorbent assay (ELISA) by Clark and Adams (1977), employed for successful detection of plant viruses, this technique has been widely adopted and modified based on requirement of the assays (Fang and Ramasamy 2015). This serological method is used for the identification of diseases based on antibodies and colour change in the assay. Serological assays depend on antibodies generated against specific antigens of plant pathogens. The antibodies bind specifically to its antigens and consequently are detected by the enzymatic digestion of substrates. Polyclonal and monoclonal antibodies have been produced against fungal antigens present in culture filtrate, cell fractions, whole cells, cell walls and extracellular components (Narayanasamy 2005).

Species of several seed-borne fungi like *Aspergillus*, *Penicillium* and *Fusarium* have been demonstrated to be potential mycotoxin producers. A monoclonal antibody (MAb) capable of reacting with antigens of 10 field fungi and 27 storage fungi was generated. The presence of fungal pathogens in barley seeds was detected using a polyclonal antibody (PAb) raised against *Penicillium aurantiogriseum* var.

melanoconidium in indirect ELISA test. A clear linear relationship was recorded between absorbance and fungal population increase, suggesting the utility of these antibodies for a broad-spectrum assay to determine the fungal content in seeds (Banks et al. 1993). Rice and corn seeds colonizing fungi, viz. *Aspergillus parasiticus*, *Penicillium citrinum* and *Fusarium oxysporum*, were detected by employing double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) test. The absorbance values of ELISA were in good correlation with concentration of mould growth, and the sensitivity of this DAS-ELISA was 1 µg/ml (Chang and Yu 1997).

Karnal bunt disease of wheat caused by *Tilletia indica* is an internationally quarantined fungal disease with a significant impact on international wheat trade as well as quality and quantity of wheat seed. SDS-PAGE analysis suggested that *T. indica* has a protein of 64 kDa weight with antigenic properties. Antibodies specific to this protein specifically reacted with pathogen's teliospores in a microwell sandwich-ELISA and dipstick immunoassay. The detection limit of both of these immunoassays was 1.25 ng/well of purified *T. indica* protein or 40 ng/well of crude spore extract, which distinguished Karnal bunt from all wheat smuts and, to some degree, the rice smut, *T. barclayana* (Kutilek et al. 2001). *Ustilago nuda* causes loose smut in barley and it is an internally seed-borne pathogen. Eibel et al. (2005b) employed a DAS-ELISA test with biotinylated detection antibodies to detect loose smut pathogen in naturally infected barley seeds.

Phomopsis longicolla is a seed-borne fungal pathogen causing *Phomopsis* seed decay of soybean, a major concern for quality seed production in soybean (*Glycine max* L.). This pathogen was detected using indirect ELISA and a modified immunoblot assay, named as seed immunoblot assay (SIBA). The comparative efficiency of both detection assays was evaluated. The problems with nonspecific interference occurred during ELISA test could be solved by employing seed immunoblot assay (SIBA) for detection. In SIBA, infected soybean seeds are transferred to nitrocellulose paper on which the mycelium of *P. longicolla* grows out forming a clearly visible coloured blotch on the nitrocellulose paper after the assay. Since the viable spores can only produce the mycelium, SIBA test is capable of differentiating the living and dead spores of the pathogen, which is a distinct advantage of this technique. In contrast ELISA test results do not offer such vital information (Gleason et al. 1987). Similarly, wheat seeds with different grades of Karnal bunt (*Tilletia indica*) infection could be readily detected by seed immunoblot binding assay (SIBA). After the immuno-processing, coloured imprints were produced on nitrocellulose paper on which infected wheat seeds were placed for vigour test, indicating the presence of viable teliospores of *Tilletia indica* in the wheat seed lots tested (Kumar et al. 1998).

Two methods, viz. PCR-based assay and DAS-ELISA, were developed and evaluated for the detection of *Tilletia caries* (syn. *T. tritici*), a seed-borne fungus causing common bunt in wheat. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was performed using biotinylated detection antibodies. The presence of bunt pathogen could be detected by PCR in shoots as well as in leaves of infected wheat plants. Except for the closely related *T. controversa*, no cross-reactions with other fungi were observed with both methods. The analysis of results obtained from DAS-ELISA of plant shoots revealed that

artificial inoculation of seeds with *T. caries* at EC 10 was efficient in infecting all host population, with a great variability in the inoculum of pathogen. ELISA employed in the assay was found most suited than PCR assay, allowing precise quantification of the amount of fungal antigen present in the plant (Eibel et al. 2005a). Agar plating method and DAS-ELISA were compared for the detection of *Macrophomina phaseolina*, a seed-borne fungal pathogen and causal agent of root rot diseases in wide host range. The presence of the pathogen was confirmed in four out of five lots by both the detection methods; however, DAS-ELISA format revealed more sensitivity in the detection of the pathogen in higher percentage of seeds as compared to agar plating method which additionally required much time and is inconvenient (Afouda et al. 2009).

Instead of successful detection of some seed-borne fungi, we may conclude that serological techniques have limited values in the detection of seed-borne fungal pathogens, since they contain many nonspecific antibodies which may cause cross-reactions with related and unrelated species concealing the effects of specific antibodies (Dewey 1992; Miller et al. 1992). These assays are widely applied to detect seed-borne viruses, but insufficiency of species-specific antibodies is a major limitation in its wide application for the detection of seed-borne fungal pathogens. Besides, serology-based assays can also detect non-viable fungal propagules, which can lead to imprecise interpretations (Mancini et al. 2016).

5.3.3 Nucleic Acid-Based Detection Methods

Generally, nucleic acid-based techniques resulting in a high level of sensitivity and specificity are used for species-specific detection of seed-borne pathogens. Through these techniques, very small quantities of samples or tissues are sufficient for the detection of pathogens in seeds of various crops. In recent times nucleic acid-based detection methods have become the preferred choice for detection, identification and quantification of seed-borne fungal pathogens. In molecular detection several strategies, viz. polymerase chain reaction (PCR), multiplex PCR, magnetic capture hybridization-PCR, Bio-PCR, loop-mediated isothermal amplification, real-time PCR and DNA barcoding, are available for the detection and identification of pathogens which involves propagation of putative pathogen propagules on a culture medium and subsequent PCR on washes from the culture plates, often using nested PCR primer pairs and sometimes without DNA extraction.

5.3.3.1 Polymerase Chain Reaction (PCR)

Molecular-based methods began after the introduction of PCR in the mid-1980s. Over the past few decades, considerable advancement has taken place in the development of molecular diagnostics for the detection of pathogens in seeds (Molouba et al. 2001). Potential benefits (e.g. rapid, same-day analysis, specific and sensitive tests) this new technology offers, make it extremely attractive. PCR is a method which enables amplification and multiplication, up to a manifold, of the target sequence of DNA. In fungi, internal transcribed spacer (ITS) region has been widely

used to design specific primers to detect the presence of seed-borne infection of fungi. PCR procedures/protocols have been developed for the detection of several seed-borne fungal pathogens associated with seeds of various commercially important crops (Mancini et al. 2016).

Rhynchosporium secalis, a causative agent of barley scald disease, overwinters in the plant debris, and this pathogen is capable of infecting barley seeds without producing conspicuous symptoms, or it may induce typical scald symptoms on seeds. A PCR-based detection method was developed, and in this assay, pathogen-specific primer pairs derived from the ITS region of rDNA of *R. secalis* were effective in detecting this pathogen in the symptomless seed infections. The detection assay revealed the presence of the diagnostic band in the symptomless seeds of susceptible cultivar (Lee et al. 2001). Further, a primer set (RS1 and RS3) derived from the internal transcribed spacer (ITS) regions of ribosomal RNA genes of this pathogen was used to quantify the inoculum of seed-borne infection caused by *Rhynchosporium secalis* in barley using competitive PCR (Lee et al. 2002).

A complex of three species, viz. *Alternaria brassicae*, *A. brassicicola* and *A. japonica*, are responsible for the black spot disease of crucifers. To restrict the transboundary spread of this disease by infected seeds, it is essential to ensure the absence of these pathogenic *Alternaria* species in seed shipments, which constitutes the disease management strategies. A PCR-based diagnostic technique was developed using specific primers developed from sequence analysis of internal transcribed spacer (ITS) regions of nuclear rDNA of *Alternaria brassicae*, *A. brassicicola* and *A. japonica.* This protocol was able to detect these pathogens in DNA extracted from seed macerates (Iacomi-Vasilescu et al. 2002). Another attempt was made to detect *Alternaria brassicae*, an important seed-borne fungal incitant of the black spot disease of crucifers using a polymerase chain reaction (PCR)-based assay. *A. brassicae*-specific primers sets were designed on the basis of the sequences of two clustered genes potentially involved in pathogenicity. The designed two sets of primers were used for conventional and real-time PCR assay. By both the detection methods, *A. brassicae* was specifically detected using DNA extracted from seed (Guillemette et al. 2004).

Rice blast disease, a serious threat to rice production, is caused by *Magnaporthe grisea*. A PCR-based detection protocol was developed using primers designed on the basis of nucleotide sequences of the *mif* 23, an infection-specific gene of *M. grisea*. The primers amplified target DNA from genetically and geographically diverse isolates of *M. grisea*, but not from DNA of other fungi tested, proving the specificity of the primers. The detection limit was ~20 pg of pathogen DNA. This PCR-based seed assay was capable in detecting *M. grisea* in rice seed lots with infestation rates as low as 0.2% (Chadha and Gopalakrishna 2006).

Septoria tritici (teleomorph, *Mycosphaerella graminicola*) is an economically important pathogen causing leaf blotch disease in wheat. *Septoria tritici*, naturally contaminating wheat seeds, was detected employing conventional PCR assay. The species-specific primers developed from strict alignment of ITS and α-tubulin sequences of *Septoria tritici* were used in the diagnostic assay. A single DNA fragment was amplified from DNA of *S. tritici*, but not from DNA of wheat seeds or other fungi selected, the detection limit being 0.5 pg of pathogen DNA (Consolo et al. 2009).

Downy mildew disease caused by biotrophic obligate oomycete *Peronospora arborescens* (Berk.) is one of the most economically important diseases of opium poppy (*Papaver somniferum* L.) worldwide. This pathogen was detected in opium poppy seeds using sensitive nested PCR assay. Two primers designed from the sequences of ITS region of rDNA improved the pathogen detection sensitivity significantly up to 1000-fold compared with single PCR employing same primers. The frequent detection of *P. arborescens* in seeds suggested the likely threat posed by this incitant for rapid spread through the seeds (Montes-Borrego et al. 2009).

The fungus *Corynespora cassiicola* is responsible for target spot disease in soybean in Brazil. This pathogen can be transmitted by seeds and is able to cause severe damage in this crop. Though early diagnostic of the disease by conventional seed testing is possible, species-level detection through these methods is time-consuming and cumbersome. A PCR-based assay was employed using specific GA4-F/GA4-R primers for the detection of *C. cassiicola* in pure culture and in soybean seeds. The pathogen could be detected in infected and inoculated seed samples at the low level of 0.25% (Sousa et al. 2016).

Species-specific detection of *Diaporthe phaseolorum* and *Phomopsis longicolla*, responsible for soybean seed decay, was achieved using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and TaqMan chemistry (Zhang et al. 1999). An ultrasonic processor was used to break the seed coats and cells, enabling the extraction of fungal DNA from soybean seeds. Three TaqMan primer/probe sets were designed, based on DNA sequences of the ITS regions of ribosomal DNA. Primer/probe set PL-5 amplified a 96 bp fragment within the ITS 1 region of *P. longicolla*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *meridionalis* and *D. phaseolorum* var. *sojae*. An 86 bp DNA fragment was obtained within the ITS 2 region of *P. longicolla* by the set PL-3, whereas set DPC-3 was able to produce 151 bp DNA fragment within the ITS 2 region of *D. phaseolorum* var. *caulivora*. The detection sensitivity of TaqMan primer/probe sets was as low as 0.15 fg (four copies) of plasmid DNA. When using PCR-RFLP for *Diaporthe* and *Phomopsis* detection, the assay was able to detect as little as 100 pg of pure DNA. However, TaqMan detection provided the fastest results of all the methods tested (Zhang et al. 1999).

Tilletia indica, the incitant of Karnal bunt disease, can be correctly identified based on morphological features but the germination of teliospores is time-consuming. A polymerase chain reaction (PCR) detection assay was employed using species-specific primers designed from rDNA-ITS region and 2.3 kb mtDNA fragment of this pathogen. The primer set from ITS region could specifically amplify 570 bp amplicon of *T. indica*, whereas primer set derived from mtDNA was able to amplify 885 bp amplicon of KB pathogen only (Thirumalaisamy et al. 2011). This assay was able to avoid delay in detection and wrong identification of closely related species of *T. indica* from wheat seed lots.

Spot blotch disease of wheat caused by *Bipolaris sorokiniana* is one of the important diseases of wheat. This disease development may take place through seed-borne infections. A quick and reliable PCR-based diagnostic assay was developed to detect *B. sorokiniana* using a pathogen-specific marker derived from genomic DNA. A PCR-amplified DNA amplicon (650 bp) was obtained in *B.*

sorokiniana isolates employing universal rice primer (URP 1F), and it was cloned in pGEMT easy vector and sequenced. A primer pair RABSF1 and RABSR2, of six primers designed based on sequences of PCR-amplified DNA amplicon, amplified a DNA sequence of 600 bp in *B. sorokiniana* isolates. The pathogen could be detected specifically in a mixed population comprising of total 74 isolates of *B. sorokiniana*, *Bipolaris* spp. and other pathogens infecting wheat and other hosts. This single DNA fragment was amplified only from DNA of *B. sorokiniana*, but not from DNA of other species of *Bipolaris* genus and other pathogenic fungi tested, suggesting the specificity of the detection assay, the detection limit being 50 pg of genomic DNA (Aggarwal et al. 2011).

Seed-borne fungal pathogen, *Alternaria radicina*, causes black rot disease of carrot. A PCR-based seed assay was developed for the detection of *A. radicina* from infested carrot seed. PCR primers used in assay were designed based on a cloned random amplified polymorphic DNA (RAPD) fragment of this pathogen. This seed assay was coupled with 5-day incubation under high humidity conditions to increase the fungal biomass. PCR amplification of the target *A. radicina* DNA sequence was improved by the addition of skim milk to the PCR reaction mixture. This PCR-based assay was able to detect the pathogen (*Alternaria radicina*), from seed lots with infestation rates as low as 0.1% (Pryor and Gilbertson 2001).

Pyrenophora graminea, a fungal incitant of leaf stripe disease of barley, is transmitted entirely by seed, and it cannot infect the leaf directly. This pathogen could be detected employing RAPD primers designed using a sequence-characterized amplified region (SCAR) approach. Out of 60 RAPD primers, a set of *P. graminea*-specific primers (PG2 F/R) was obtained that amplified a single DNA fragment (435 bp) from 37 isolates of *P. graminea* tested, but not from other *Pyrenophora* spp. or saprophytes isolated from barley seed. The diagnostic assay was completed within 25 min (including melting point analysis) using a LightCycler, capable of measuring emission of fluorescence from the binding of SYBR Green I dye to the PCR products. The rapidity was coupled with the closed 'in-tube' detection of PCR products which reduces the chances for contamination (Taylor et al. 2001b).

Anthracnose is mainly a seed-borne disease caused by *Colletotrichum lindemuthianum* in bean (*Phaseolus vulgaris*). The pathogen could be detected employing a rapid, specific and sensitive PCR-based detection method. Based on data analysis of sequences of rDNA region consisting of the 5.8S gene and internal transcribed spacers (ITS) 1 and 2 of 4 *C. lindemuthianum* races and 17 *Colletotrichum* spp. downloaded from GenBank, 5 forward primers were designed. One forward primer showing specificity of the detection was selected for use in combination with ITS 4 to specifically detect *C. lindemuthianum*. A 461 bp specific DNA band was obtained from the genomic DNA template of 16 isolates of *C. lindemuthianum*, but not from other *Colletotrichum* species or 10 bean pathogens. A nested PCR protocol was applied to enhance the sensitivity of detection, which enabled the detection of as little as 10 fg of *C. lindemuthianum* genomic DNA and 1% infected seed powder. This detection assay could be accomplished within 24 h against a 2-week period required for culturing the pathogen, and this protocol required no specialized taxonomic expertise (Fig. 5.4) (Chen et al. 2007).

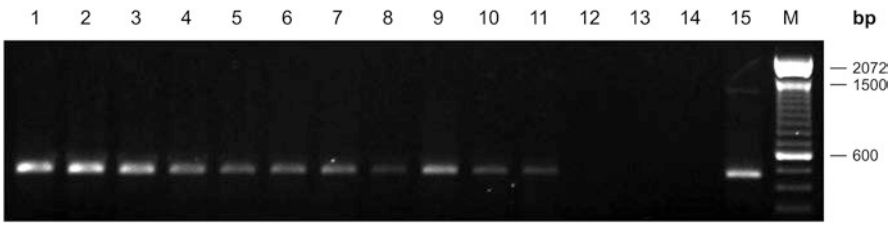

Fig. 5.4 Detection of *Colletotrichum lindemuthianum*-specific DNA fragment in bean seed powder using nested PCR assay. Lanes: M, 100 bp DNA ladder (Invitrogen); 1, 100%; 2, 80%; 3, 60%; 4, 40%; 5, 20%; 6, 10%; 7, 8%; 8, 6%; 9, 4%; 10, 2%; 11, 1%; 12, 0%; 13, anthracnose-resistant bean genotype G2333; 14, negative control (water replaced genomic DNA as the template); 15, positive control (*C. lindemuthianum* DNA). (Courtesy of Chen et al. 2007)

Fusarium wilt of lettuce (*Lactuca sativa*) is a serious threat for the lettuce production around the world. The fungal incitant of the disease *Fusarium oxysporum* f. sp. *lactucae* has the potential for seed-borne spread. *Fusarium oxysporum* f. sp. *lactucae* was detected in the seed of lettuce using a nested polymerase chain reaction (nPCR)-based assay. Sequences of intergenic spacer region of the rDNA were used to design three primers for PCR amplifications. A PCR product (2270 bp) was generated using primer pair GYCF1 and GYCR4C in the first amplification. A 936 bp DNA fragment was amplified employing the forward primer GYCF1 and the nested primer R943 in the second amplification. The nPCR protocol successfully detected the target sequence in genomic DNA of *Fusarium oxysporum* f. sp. *lactucae* at 1 fg/μl. The nPCR seed assay was coupled with a 4-day incubation under high humidity conditions to increase fungal biomass for DNA extraction. In seed lots known amounts of *F. oxysporum* f. sp. *lactucae*-infested seed were mixed with non-infested seed, and this assay detected the pathogen from seed lots with infestation rates as low as 0.1% (Mbofung and Pryor 2010).

5.3.3.2 Multiplex PCR

Multiplex polymerase chain reaction (multiplex PCR) is a valuable molecular tool which offers simultaneous amplification of several DNA amplicons of different sizes, within single PCR reaction (Sint et al. 2012). The multiplex PCR technique was first described by Chamberlain et al. (1988). This technique has various applications and is being commonly used for the identification and detection of pathogens, gene deletion analysis, high-throughput SNP genotyping, linkage and mutation analyses, etc. Since multiplex PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of different sizes that are specific to different DNA sequences, it is capable of detecting several seed-borne pathogenic fungi simultaneously with high sensitivity. Preferably, potential seed health tests can be designed as multiplex assays for particular crops, with their ability to detect all seed-borne pathogens required for phytosanitary purposes.

Multiplex PCR was employed to differentiate members of two groups belonging to *Aspergillus flavus.* The first *Aspergillus flavus* group encloses *A. flavus* and *A. parasiticus* as aflatoxin producers, and the second group includes *A. oryzae* and *A.*

sojae which are best known for their capability to ferment soybean to prepare various food products. Aflatoxigenic strains could be detected, and their differentiation was possible employing the multiplex PCR protocol that minimized the risk of a genotype being a phenotypic producer of aflatoxin. This assay was based on four genes involved in aflatoxin biosynthesis, viz. norsolorinic acid reductase (nor-1), versicolorin A dehydrogenase (ver-1), sterigmatocystin O-methyltransferase (omt-1) and a regulatory protein (apa-2) (Chen et al. 2002). Multiplex PCR could successfully detect *Fusarium* species within *Fusarium* head scab complex (Waalwijk et al. 2003) and *Rhynchosporium secalis*, a seed-borne fungal incitant causing economically important leaf blotch disease of barley (Fountaine et al. 2007). There may be chances that a seed may harbour very closely related species of fungi. The occurrence of very closely related fungal species on the seed may lead to overlapping while simultaneous detection. In multiplex PCR, possible problem of overlapping of amplicons with similar sizes could be overcome using primers, already dyed with different colour fluorescent dyes (Sint et al. 2012).

Comparison of two PCR-based protocols was done for the detection of *Fusarium verticillioides* and *Fusarium subglutinans*, important fungal pathogens of maize and other cereals worldwide. PCR-based protocols were used for the identification of these pathogens targeting the *gaoB* gene, which codes for galactose oxidase. The designed primers recognized isolates of *F. verticillioides* and *F. subglutinans* obtained from maize seeds from several regions of Brazil but did not recognize other *Fusarium* spp. or other fungal genera. A multiplex PCR diagnostic protocol was capable to simultaneously detect the genomic DNA from *F. verticillioides* and *F. subglutinans* growing in artificially or naturally infected maize seeds (Faria et al. 2012).

Fusarium culmorum causes disease complex, viz. seed rot, seedling blight and ear rot in maize. The multiplex PCR assay was standardized targeting trichothecene metabolic pathway genes, viz. *Tri6*, *Tri7* and *Tri13*, for the detection of trichothecene (DON/NIV) chemotypes and rDNA gene for the specific detection of *Fusarium culmorum* species in freshly harvested maize seeds. The analysis of primers employed in multiplex PCR assay revealed that 94 isolates were able to produce deoxynivalenol/nivalenol DON/NIV. The practical usefulness of mPCR assay was validated by comparing these results with high-performance thin-layer chromatography (HPTLC) and found that mPCR results equivocally matched with the HPTLC chemical analysis for field samples (Venkataramana et al. 2013).

5.3.3.3 Magnetic Capture Hybridization (MCH)-PCR

Interference caused by inhibitory compounds of seed extracts in conventional PCR is a major limitation affecting both assay sensitivity and reliability. The magnetic capture hybridization-PCR (MCH-PCR) has the advantage that the MCH process purifies and concentrates the DNA of interest while removing non-target DNA and other substances that can inhibit the in vitro enzymatic manipulation of nucleic acids that are normally found in complex sharing biological material. In MCH-PCR, magnetic beads coated with single-stranded DNA probes are used to capture DNA fragments which are further used for PCR amplification. This technique has been successfully used to detect fungi, viruses and bacteria in materials containing

PCR inhibitory compounds (Jacobsen 1995). With the use of MCH-PCR, the detection sensitivity can be enhanced up to 10- to 100-fold as compared to conventional PCR protocol.

Complex of three species of *Botrytis*, viz. *B. aclada*, *B. allii* and *B. byssoidea*, are responsible for neck rot disease in onion. The pathogens of this disease are transmitted by onion seeds. An MCH-PCR diagnostic protocol was employed for the rapid and sensitive detection of *B. aclada* in onion seed samples. The DNA of *B. aclada* could be detected using MCH-PCR diagnostic protocol, in aqueous solutions prepared from seed extract with detection limit as 100 fg of fungal DNA/ml. MCH-PCR protocol was more sensitive and efficient than normal PCR, in detecting the fungus *B. aclada* in seed lots with 4.8% and 9.9% infection in naturally infested seeds. MCH-PCR detection assay could be completed within 24 hours against a 10–14-days period required in conventional methods to test onion seeds (Walcott et al. 2004). Two important seed-borne pathogens of cucurbits could be detected by employing a magnetic capture hybridization (MCH) multiplex real-time PCR assay. This assay offered the improved simultaneous detection of two different pathogens in cucurbit seed lots, viz. *Acidovorax avenae* subsp. *citrulli*, a causal agent of bacterial fruit blotch, and *Didymella bryoniae*, a fungal incitant of gummy stem blight disease (Ha et al. 2009).

5.3.3.4 Bio-PCR

Bio-PCR enables the enhancement of fungal biomass since seed-borne fungi generally infect the host seeds at very low concentration of inoculum, and therefore the DNA of the fungal pathogen is not enough for the subsequent reactions, limiting the use of conventional PCR-based detection. This technique was developed by Schaad et al. (1995) primarily to detect a seed-borne bacterium from bean seed extracts. Later, this technique was also proved to be efficient for detection of fungi (Munkvold 2009). Bio-PCR is mainly applied for fungi and bacteria. In this detection method, a pre-assay incubation step is coupled with PCR process. Bio-PCR consists of the preventive growth of non-target pathogens on selective medium and selective increase in the biomass of target microorganisms, followed by DNA extraction and amplification by PCR (Schaad et al. 1995).

Bio-PCR has been proven successful in the detection/identification of *Tilletia indica* teliospores in wheat seed samples (Schaad et al. 1997). In Bio-PCR, standard deep-freeze blotter method was utilized as pre-assay incubation to increase the fungal biomass of *Alternaria dauci* and *A. radicina* from infected seeds of carrot and was detected using of specific primers of *A. dauci* and *A. radicina* during the PCR assay (Konstantinova et al. 2002).

Though Bio-PCR offers several advantages over conventional PCR assay, like it is highly sensitive, eliminates PCR inhibitors and avoids false positives due to dead cells since it detects live cells only (Marcinkowska 2002), there are some limitations of Bio-PCR also, viz. if selective media are used, the method becomes more expensive and pre-assay requires 5–7-days period to increase fungal growth, which substantially increases the time required for the completion of the assays (Mancini et al. 2016).

5.3.3.5 Loop-Mediated Isothermal Amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is one of the novel nucleic acid amplification technologies that enables the synthesis of large amounts of DNA in a short period of time with high specificity. This technique was developed by Notomi et al. (2000) as a simple, cost-effective and rapid method for the specific detection of genomic DNA (Mancini et al. 2016). In the future it may be a potential alternative to PCR, since LAMP protocol does not require thermocycler apparatus. LAMP uses a pair of four or six oligonucleotide primers with eight binding sites hybridizing specifically to diverse areas of a target gene and a thermophilic DNA polymerase from *Geobacillus stearothermophilus* for DNA amplification. Additionally, being a highly specific diagnostic protocol, the amplification efficiency of LAMP is extremely high, which provides improved sensitivity, and can overcome the problem of inhibitors that usually adversely affect PCR methods (Fu et al. 2011). LAMP products can be visualized by gel electrophoresis, using magnesium pyrophosphate, which enhances precipitation of amplified DNA (Fukuta et al. 2003; Nie 2005), with a real-time turbidity reader (Fukuta et al. 2004; Mori et al. 2004), or with the addition of an intercalating dye, such as SYBR Green I, which produces a colour change in the presence of target phytopathogen (Iwamoto et al. 2003; Mumford et al. 2006).

Fusarium graminearum is the major causative agent among the species complex of *Fusarium* head blight of small cereals and is potential producer of the mycotoxins, viz. deoxynivalenol, nivalenol and zearalenone. The pathogen could be detected by employing LAMP assay, based on the *gaoA* gene (galactose oxidase) of *Fusarium graminearum*. Amplification of DNA during the reaction was indirectly detected in situ by using calcein fluorescence as a marker, circumventing the use of time-requiring electrophoretic analysis. The LAMP protocol was able to detect the presence ~2 pg of purified target DNA per reaction within 30 min, specifically from DNA of *F. graminearum* (Niessen and Vogel 2010).

Fusarium oxysporum f. sp. *ciceris* (Foc), the incitant of *Fusarium* wilt, is both a soil-borne and seed-borne fungus. It is one of the most devastating pathogens of chickpea, causing major economic losses ranging from 10% to 40% worldwide. It is estimated to cause a 10–15% yield loss annually in India (Haware and Nene 1982). A loop-mediated isothermal amplification (LAMP) assay was developed targeting the elongation factor 1 alpha gene sequence for visual detection of Foc. The LAMP reaction was optimal at 63 °C for 60 min. In the presence of hydroxynaphthol blue (HNB) added before amplification, DNA of Foc developed a characteristic sky blue colour, whereas this colour was absent in the DNA of six other plant pathogenic fungi. Later, gel electrophoresis analysis confirmed the results obtained with LAMP and HNB. The detection limit of this LAMP assay for Foc was 10 fg of genomic DNA per reaction, against 100 pg of conventional PCR (Ghosh et al. 2015).

Karnal bunt in wheat caused by a fungus *Tilletia indica* is a quarantine disease, and therefore timely and specific detection of the pathogen is very essential. The pathogen could be detected specifically with rapidity employing the loop-mediated isothermal amplification (LAMP) at 62 °C. Four major unique regions were identified in *T. indica* through analysis of alignment of the mitochondrial DNA of *T. indica*

and *T. walkeri.* Six LAMP primers designed from one of these major unique regions in *T. indica* could amplify *T. indica* DNA. Among 17 isolates of *T. indica*, *T. walkeri*, *T. horrida*, *T. ehrhartae* and *T. caries*, this protocol offered highly specific detection of *T. indica.* Endpoint detection with the naked eye could be possible using the fluorescent chemical calcein. The diagnostic assay could be completed in 30 min, offering similar sensitivity as with conventional PCR. The specificity issues that occurred during PCR-based detection protocols due to the high DNA homology of *T. indica* with other *Tilletia* species, especially *T. walkeri*, could be solved by employing this technique for detection (Gao et al. 2016).

Phomopsis longicolla is an important seed-borne fungal pathogen responsible for the deterioration in the seed quality of soybean. The pathogen could be detected employing loop-mediated isothermal amplification (LAMP) diagnostic assay based on transcription elongation factor 1-α (*TEF1-α*), identified as a suitable target for the detection of *P. longicolla.* This LAMP diagnostic assay, with great specificity, was capable to detect all 54 isolates of *P. longicolla* from the rest of the 41 isolates of other fungi tested. Before the amplification of LAMP products, hydroxynaphthol blue (HNB) was added, and a sky blue colour was only developed in the presence of *P. longicolla*, while other fungal isolates failed to show colour change. The detection limit of the assay was 100 pg/μL fungal DNA, and additionally the assay also detected *P. longicolla* from diseased soybean tissues and residues from different origins (Dai et al. 2016).

Anthracnose is a worldwide occurring fungal disease of soybean. This disease is primarily caused by *Colletotrichum truncatum.* Rapid and direct detection of the pathogen in diseased soybean tissues could be possible employing a loop-mediated isothermal amplification (LAMP) assay. A pair of species-specific primers was designed using the target gene *Rpb1* (that codes for the large subunit of RNA polymerase II). During the screening, species-specific primers amplified the genomic DNA of *Colletotrichum truncatum* at 62 °C over 70 min. The presence of *C. truncatum* could be confirmed by a yellow-green colour (visible to the unaided eye), developed in LAMP reaction products after addition of SYBR Green I dye. This *Rpb1*-Ct-LAMP assay could successfully diagnose soybean anthracnose in field samples collected from various locations of China and was able to detect *C. truncatum* in soybean seeds from farmers' markets, the detection limit being 100 pg (per μL genomic DNA of pathogen) (Tian et al. 2017).

5.3.3.6 Real-Time PCR

It is a laboratory technique of molecular biology based on PCR, which consists of amplification and simultaneous detection or quantification of targeted DNA molecule. Real-time PCR consists of coupling DNA amplification with fluorescent substances which can be easily measured, giving an indirect measurement of DNA amplification. This is the case of TaqMan (Heid et al. 1996; Taylor et al. 2001a) where fluorescence is directly linked to the excision of reporter dye molecules, which is directly related to DNA amplification. In contrast to other detection techniques, a much quicker and more sensitive, quantitative assay could be provided by real-time PCR assays.

Detection through real-time PCR was reported for *Didymella bryoniae*, an incitant of gummy stem blight of cucurbits (Ling et al. 2010). A real-time fluorescent polymerase chain reaction (PCR) assay was developed using SYBR Green chemistry to quantify three species of *Botrytis*, viz. *B. aclada*, *B. allii* and *B. byssoidea*, associated with onion (*Allium cepa*) seed that are also able to induce neck rot of onion bulbs (Chilvers et al. 2007). The nuclear ribosomal intergenic spacer (IGS) regions of target and non-target *Botrytis* spp. were sequenced and aligned and used to design a primer pair specific to *B. aclada*, *B. allii* and *B. byssoidea.* The primers reliably detected 10 fg of genomic DNA per PCR reaction extracted from pure cultures of *B. aclada* and *B. allii* (Chilvers et al. 2007).

Simultaneous detection of *Pantoea ananatis* and *Botrytis allii* was performed in onion seeds using magnetic capture hybridization and real-time PCR (Ha and Walcott 2008). Montes-Borrego et al. (2011) have achieved real-time PCR quantification of *Peronospora arborescens*, the opium poppy downy mildew pathogen, in seed stocks and symptomless infected plants. Ioos et al. (2012) have used duplex real-time PCR tool for sensitive detection of the quarantine oomycete *Plasmopara halstedii* in sunflower seeds. A real-time PCR assay utilizing SYBR Green was developed to detect *V. dahliae* associated with spinach seed (Duressa et al. 2012). More recently, a multiplex TaqMan real-time PCR assay was developed for the detection of spinach seed-borne pathogens, viz. *Peronospora farinosa* f. sp. *spinaciae*, *Stemphylium botryosum*, *Verticillium dahliae* and *Cladosporium variabile*, that cause economically important diseases on spinach (Feng et al. 2014). They tested TaqMan assays on DNA extracted from numerous isolates of the four target pathogens, as well as a wide range of non-target, related fungi or oomycetes and numerous saprophytes commonly found on spinach seed. Multiplex real-time PCR assays were evaluated by detecting two or three target pathogens simultaneously. Singular and multiplex real-time PCR assays were also applied to DNA extracted from bulked seed and single spinach seed (Feng et al. 2014). *Fusarium oxysporum* f. sp. *phaseoli* is a devastating pathogen that can cause significant economic losses and can be introduced into fields through infested common bean (*Phaseolus vulgaris*) seeds. Robust seed health testing methods can be helpful in preventing long-distance dissemination of this pathogen by contaminated seeds. A rapid real-time PCR assay (qPCR) protocol was developed for the detection and quantification of *Fusarium oxysporum* f. sp. *phaseoli* in common bean seeds. SYBR Green and TaqMan qPCR methods were compared directly using primers based on the Fop virulence factor *ftf1*. Both qPCR assays detected infection in seed at low levels (0.25%); however, the TaqMan assay was found more reliable at quantification than the SYBR Green assay (Sousa et al. 2015). 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 to design the primers and probes (Mancini et al. 2016). Recently, a real-time PCR-based marker was developed for the detection of teliospores of *Tilletia indica* in soil (Gurjar et al. 2017).

5.3.3.7 DNA Barcoding

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species (Hebert et al. 2003). The nuclear ribosomal internal transcribed spacer (ITS) region is a recently proposed DNA barcode marker for fungi (Schoch et al. 2012). Identification of universal barcoding regions is important to detect seed-borne fungi. Internal transcribed spacer (ITS) has been used as the primary barcode marker for fungi based on its ability to successfully identify inter- and intraspecific variation among a wide range of fungi. An ideal barcoding gene should be sufficiently conserved to be amplified with wide range of primers, however divergent enough to identify closely related species. Other applications include, for example, identifying plant leaves even when flowers or fruits are not available and identifying insect larvae (which may have fewer diagnostic characters than adults and are frequently not well-known) (Kress et al. 2005). Barcoding regions of some important seed-borne fungi are given in Table 5.3.

5.3.4 Next-Generation Sequencing (NGS)

After its first application in basic biological research, NGS technologies have been extended to other fields of application, which have included plant disease diagnosis. As NGS has been a valuable technique for the 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 and 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 and Marra 2008).

Illumina/Solexa sequencing is similar to the Sanger-based methods, because it uses terminator nucleotides incorporated by a DNA polymerase. However, Solexa

Table 5.3 Barcoding regions of some seed-borne fungi

S. no.	Barcoding regions	Seed-borne fungi	References
1.	Translation elongation factor-1 alpha (TEF-1 alpha)	*Fusarium* spp.	Amatulli et al. (2010)
2.	Intergenic spacer sequence (IGS)	*Fusarium verticillioides*	González-Jaen et al. (2004)
3.	Internal transcribed spacers (ITS) region	*Alternaria alternata*, *A. infectoria* and *A. triticina*	Links et al. (2014)
		Peronospora arborescens	Landa et al. (2007)
		Albugo candida	Robideau et al. (2011)
		Pseudoperonospora cubensis	Robideau et al. (2011)
4.	Lpv gene	*Phytophthora cinnamoni*	Kong et al. (2003)
5.	*Cytochrome* C oxidase 1	*Aspergillus* spp.	Geiser et al. (2007)
		Albugo candida	Robideau et al. (2011)
		Penicillium spp.	Seifert et al. (2007)
		Pseudoperonospora cubensis	Robideau et al. (2011)
6.	Pot2 transposon	*Magnaporthe oryzae*	Kachroo et al. (1994)
7.	LSU regions	*Albugo candida*	Robideau et al. (2011)
		Pseudoperonospora cubensis	Robideau et al. (2011)
8.	α-Tubulin sequences	*Septoria tritici* and *Rhynchosporium secalis*	Rohel et al. (1998)
9.	NADH dehydrogenase	*Fusarium* sp.	Kamil et al. (2015)

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 be proved useful in the future for routine seed diagnosis.

5.3.5 Other Newly Developed Diagnostic Techniques

5.3.5.1 Biospeckle Laser Technique

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.

5.3.5.2 Videometer Lab Instrument

One more recently developed tool called videometer lab instrument that can distinguish infected seeds from healthy seeds is a multispectral vision system also useful to determine the colour, texture and chemical composition of seed surfaces (Boelt et al. 2018). The combinations of the features from images captured by visible light wavelengths and near-infrared wavelengths were worthwhile 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). Seed quality of castor (*Ricinus communis* L.) based on seed coat colour was predicted employing multispectral imaging technology using VideometerLab instrument. This technology was able to distinguish viable seeds from dead seeds with 92% accuracy, suggesting its utility for seed deterioration caused by fungal pathogens (Olesen et al. 2015).

5.4 Summary

Seed health has become an important quarantine issue, mainly in the international movement of seeds and germplasm exchange. Thus, it is essential to make sure that no potentially damaging pathogens are established on seeds. Conventional seed detection methods including visual examination, selective media, seedling grow-out assay and the serological assays have been used extensively, but all have limitations like inefficiency and sensitivity. The molecular methods have shown great potential for improving pathogen detection in seeds as it embodies many of the key characteristics including specificity, sensitivity, rapidity, ease of implementation, interpretation and applicability. PCR and its modifications including Bio-PCR and MCH-PCR may offer opportunities to evade inhibitory compounds while improving detection of seed-borne pathogens. Further, reduced cost and more efficiency will ultimately allow DNA-based detection methods to replace the vast range of seed detection assays presently engaged and will provide advanced detection abilities essential for healthy seedling establishment. A comparative analysis of various seed detection methods based on the time required for completion, sensitivity, ease of application and specificity along with the examples of fungi detected on seeds using the particular technique has been summarized below in Table 5.4.

Table 5.4 General features of seed detection assays including the time required for completion, sensitivity, ease of application, specificity and applicability for the detection of fungi on seed (Walcott 2003; Mancini et al. 2016)

Type of assay	Time required	Sensitivity	Ease of application	Specificity	Ease of implementation	Examples
Visual examination	5–10 min	Low	Simple and inexpensive (requires experience)	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	10–30 min	Low	Simple and inexpensive	Low	Mycological skills required	*Peronospora manshurica*/soybean seed
Semi-selective media	2–14 days	Moderate	Simple and inexpensive	Low–moderate	Mycological skills required	*Alternaria brassicicola* (crucifer seeds)
Seedling grow-out assay	2–3 weeks	Low	Simple, inexpensive and robust	Low	Mycological skills required	Fungal seedling diseases caused by *Alternaria*, *Bipolaris*, *Fusarium*, *Pyricularia*, etc.
Freeze blotter incubation	1 week	Low/moderate	Simple and inexpensive	Moderate	Mycological skills required	*Alternaria dauci*, *Alternaria radicina*/carrot seed; *Leptosphaeria maculans*/Brassicaceae seed
Agar medium incubation	5–7 days	Low/moderate	Simple and inexpensive	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	2–4 h	Moderate–high	Simple, moderately expensive and robust	Moderate–high	Ease of interpretation	*Macrophomina phaseolina*/cowpea seed

(continued)

Table 5.4 (continued)

Type of assay	Time required	Sensitivity	Ease of application	Specificity	Ease of implementation	Examples
Conventional DNA extraction and PCR	5–6 h	High	Complicated; easy to interpret, expensive	Very 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 (selective target colony enrichment followed by PCR)	5–7 days	Very high	Complicated, expensive	Very 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
MCH-PCR (magnetic capture hybridization and PCR)	2–5 h	Very high	Complicated, expensive	Very high	Molecular biology skills required	*Didymella bryoniae*/Cucurbitaceae seed; *Botrytis* spp./onion seed
Nested PCR	5–6 h	Very high	Complicated, expensive	High	Molecular biology skills required, ease of interpretation	*Colletotrichum lindemuthianum*/bean seeds; *Fusarium oxysporum* f. sp. *lactucae*/lettuce seeds
Real-time PCR	40–60 min	Very high	Complicated, expensive	Very 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
DNA microarrays	6 hours	Very high	Complicated, expensive	Very high	Molecular biology skills required	*Botrytis cinerea*, *B. squamosa* and *Didymella bryoniae*
Laser biospeckle technique	High	High	Complicated, expensive	High	Technological skills required	*Fusarium oxysporum*, *Aspergillus flavus*, *Sclerotinia* spp./bean seed
Videometer	High	High	Complicated, expensive	High	Technological skills required	*Stemphylium botryosum*, *Cladosporium* spp., *Fusarium* spp., *Verticillium* spp., *Alternaria alternata*/spinach seed

5.5 Challenges and Future Directions

Besides the unique advantages offered by the various seed/plant disease detection methods, each method has its own limitations. 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. 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.

5.6 Conclusion

In this chapter, we reviewed the currently existing methods for detection of seed-borne fungal phytopathogens. Although the conventional methods are widely used for the detection of seed-borne fungal phytopathogen presently, they are relatively difficult to operate, require expert technicians and are time-consuming for data analysis. Ultimately, improved protocols based upon PCR, ELISA, etc. will be available for the detection of all seed-borne pathogens and may supersede conventional detection methods.

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