Detection and diagnosis of seed-borne pathogens

Introduction

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.

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:

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.

Importance of Detection of Seed-Borne Fungal Pathogens

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.

Detection Methods for Seed-Borne Fungal Pathogens

I. Conventional Detection Methods

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. 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.

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

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.

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 is used for detecting loose smut pathogen *Ustilago nuda* var. *tritici*. In the method, embryos are dissected, macerated with NaOH and then stained with aniline blue. This method is completed in 3–4 days.

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:

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:

1. 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 for a couple of days depending on the fungus and type of seed tested. 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. 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 detected.

2. 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.

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. 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:

1. 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.

2. Hiltner's Brick Stone Method

3. Sand Method

4. Standard Soil Method

d. Selective Media

4. Serological Detection Techniques

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. 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.

5. 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.