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CHAPTER

Detection of Seedborne Pathogens

7.1 DETECTION OF FUNGAL PATHOGENS

Normally, the seed analysts are well versed with the procedures involved in testing the seed samples for purity analysis, germination and moisture content etc., but they are almost ignorant about the procedures involved in seed health testing. So, an attempt has been made to describe in detail the various steps involved in analyzing the seed samples for seed health. Accordingly, for determining the health status of a seed sample the analysis may be done in following steps :

7.1.1 Examination of dry seeds

The first step in seed health testing is the examination of the dry seeds with naked eye or with the help of a hand lens. A number of seedborne pathogens often produce a discolouration on seed surface. Even saprophytes or weak pathogens may produce a discolouration which reduces the commercial value of the seed particularly when it is to be used for human consumption. As per International Seed Testing Association Rules (Anon, 2008) the entire submitted sample, or a portion of it, depending on the test method, may be used as a working sample for seed health. For examination of dry seeds, entire seed sample should be examined as it is a non-destructive method of seed health testing. However, the analyst should know what he will be looking for, in examination of dry seeds for health testing. There are various types of symptoms that the pathogen produces on the seed surface. These symptoms have been described in following pages.

Neergaard (1979) classified these symptoms into different groups, which are as follows:

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7.1.1.1 Seed rot

A number of fungi associated with seeds cause seed rot during germination. Some of the fungi causing seed rot are listed below:

- Fusarium avenaceum*, *F. culmorum*, *F. graminearum* and *F. moniliforme* cause seed rot in cereals.
- Fusarium semitectum* causes dry rot in rice grains.
- Drechslera oryzae* in rice,
- Drechslera maydis* in maize and
- Drechslera sorokiniana* in other cereal crops also causes seed rot.
- Tricochoniella padwickii* in rice,
- Curvularia penniseti* in pearl millet,
- Alternaria porri* f.sp. *dauci* and *A. radicina* in carrot
- Colletotrichum graminicola* in soybean,
- Phoma lingam* in cabbage,
- Mycosphaerella pinnodes* in pea and
- Diaporthe phaseolorum* in soybean also causes rot on seeds.

7.1.1.2 Seed necroses

Most of the seedborne pathogens produce superficial symptoms on seed surface, and are restricted only upto seed-coat or the pericarp of the seed. However, the species of *Colletotrichum* and *Ascochyta* causing anthracnose in seeds of soybean, pea and cowpea often penetrate deep into the fleshy cotyledons producing necrotic lesions.

7.1.1.3 Shrunken seeds

The seed size may be reduced either due to fungal infection or may be due to poor development of the seed. Many pathogens cause reduction in seed size. e.g.

- Septoria nodorum* in wheat
- Drechslera teres* in barley
- Alternaria brassicicola* in crucifers
- Phoma lingam* in crucifers
- Septoria linicola* in flax
- Pemospora destructor* in onion

Sclerophthora macrospora in maize
Sclerospora philippinensis in maize
Sclerospora sorghi in sorghum
Albugo candida in crucifers
Puccinia helianthi in sunflower

7.1.1.4 Seed Abortion

Some parasitic fungi attack the young seed primordia and the flower structures. They cause deterioration of ovule and thus reduces the seed yield. e.g.

Gloeotinia temulenta causes Blind Seed in ryegrass
Fusarium culmorum and *F. graminearum* in wheat and rice
Fusarium moniliforme in maize

Ascochyta rabiei kills the young seed in chickpea
Podosporella verticillata causes death of seed primordia in wheat. The disease is known as Sterility Disease.

7.1.1.5 Seed discolouration

Seed discolouration indicate that seed transmitted pathogen is present in the seed, though this may not necessarily be so. If the seed is to be used for consumption, discolouration tells the poor quality of the seed.

In many cases discolouration may not affect the seed quality but it definitely reduces the market value. The discolouration may be of three types:

7.1.1.5.1 Superficial necrotic lesions A large number of seedborne pathogens are known to produce black, brown or grey necrotic lesions on the seed-coat. e.g.

Ascochyta pisi on pea
Colletotrichum lindemuthianum in beans
Drechslera oryzae in rice (Pecky rice)
Fusarium equiseti, *F. semitectum* and *Macrophomina phaseolina* in mungbean
 Dark brown or black spots in sorghum due to *Colletotrichum graminicola* and *Phoma* spp.

White streaks radiating from the embryo region of maize seed is due to *Fusarium moniliforme*

Black streaks radiating from the embryo region of maize seed is due to *Drechslera maydis*
 Black point or kernel smudge of wheat due to *Alternaria alternata*, *Curvularia lunata*, *Drechslera sativa* etc.

7.1.1.5.2 Fungus coating Many seedborne pathogens or even saprophytes associated with seed often show profuse mycelial growth on seed surface. In cereals this type of contamination is quite common. e.g.

Drechslera sorokiniana in cereals
Drechslera teres in barley
Drechslera oryzae in rice
Alternaria tenuis
Cladosporium cladosporioides
 Oospore crust of *Sclerospora sorghi* on seed surface and glumes of sorghum.
Fusarium graminearum often makes the wheat seeds pink or orange.

7.1.1.5.3 Pigmentation Sometimes the pathogen or the saprophyte produces pigmentation on seed surface. This pigmentation is produced by the interaction of the toxins produced by the pathogen or the saprophytes and the chemicals or enzymes present in the seed. e.g.

Purple stain produced by *Cercospora kikuchii* in soybean.
Fusarium moniliforme produces a pink pigmentation in maize seeds.
Stemphylium botryosum produces a red pigmentation in the micropylar region of bean seeds. It is known as "Red Nose".
Trichoconiella padwickii produces a pink pigmentation in rice.
Alternaria tenuis produces a blue pigmentation on the lint in cotton.

7.1.1.6. Fructification

The seeds of coriander become hypertrophied due to the infection of *Protomyces macrosporus*. Species of *Phoma* and *Macrophomina* produce fructifications on the surface of seeds. Johnson and Lefebure (1942) for the first time observed the crusts of oospores of *Peronospora manchurica* on the surface of soybean seeds and concluded that downy mildew in soybean is seedborne. The seed transmission of the pathogen was established by Jones and Torrie (1946).

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Drechslera teres in barley

Drechslera oryzae in rice

Alternaria tenuis

Cladosporium cladosporioides

Oospore crust of *Sclerospora sorghi* on seed surface and glumes of sorghum.

Fusarium graminearum often makes the wheat seeds pink or orange.

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7.1.1.7 Sclerotisation

The ovary gets converted into a hard structure known as sclerotia. Species of *Claviceps* often produce sclerotia in majority of cereals and other grasses. The shape and size of the sclerotia depends largely on the host. The sclerotia of *Claviceps purpurea* produced on *Secale cereale* are stout and upto 2-3cm long, while the sclerotia of the same pathogen on *Phleum pratense*, a grass are very tiny. The Sclerotia of *Claviceps fusiformis* causing the ergot in pearl millet are rounded at one end and pointed at the other end. They look like a tiny chickpea seed. The sclerotia of *Claviceps sorghi* causing the ergot in sorghum are slender in shape.

7.1.1.8. Seed discolouration

Fungal infection on seed-coat in many crops causes discolouration which not only results in poor seed quality but also in reduced market value. Some pathogens cause black, brown or grey necrotic lesions. Ram Nath *et. al.* (1970) described these symptoms on the seeds of *Phaseolus aureus* infected with *Fusarium equiseti*, *F. semitectum* and *Macrophomina phaseolina*. The white streaks radiating from the embryo part of maize seeds is often associated with *F. moniliforme*, while the black streaks indicate the presence of *Drechslera maydis*. Heavy sporulation of *Fusarium graminearum* causing head blight in wheat also makes the kernels pink or orange in colour. Some of the pathogens or even the saprophytes produce pigmentation on the surface of the seeds, which indicates their presence. However, the ability of the fungus to produce pigmentation varies from strain to strain. It is also not necessary that all the infected seeds show pigmentation. Ou (1972) reported that rice seeds infected with *Tricothionella padwickii* give a pink stain. Brinkman (1931) observed a red discolouration around the micropyle of bean seeds infected with *Stemphyllum botryosum*. This has been termed as 'Red Nose'. Kilpatrick (1957), Johnson and Jones (1962) and Laviolette and Athow (1972) observed purple stain on the surface of soybean and clusterbean seeds due to *Cercospora kikuchii*. Similarly, *C. sojina* results in gray or brown discolouration of soybean seeds-coat. Likewise, *Macrophomina phaseolina* (black spots) and *Colletotrichum truncatum* (grayish discolouration with minute black specks) also occur on soybean seeds. The brown spots on the seeds of pea and beans often indicate the presence of *Ascochyta pisi* and *Colletotrichum lindemuthianum*. Dastur (1932) reported the blackening of the germinal end of wheat seeds due to *Alternaria tenuis*. This disease is commonly known as Black point of wheat. *Drechslera sorokiniana* and

Curvularia lunata are also associated with black point of wheat. Gaur et al (1990) reported that the seeds obtained from the partially proliferated ear-heads of pearl millet not only lose their original luster but also developed a violet tinge and are also slightly bigger in size than the healthy seeds but have a low seed weight.

7.1.1.9 Some Specific Symptoms

7.1.1.9.1 Seeds of *Coriander sativum* infected with *Protomyces macrosporus* show hypertrophy of seeds. Though this is not at all a seedborne or seed transmitted pathogen.

7.1.1.9.2 Cotyledons of pea often show the symptoms of "Hollow Heart" caused by sudden drying of the crop. It is not a disease.

7.1.1.9.3 Cotyledons of pea may also show "Marsh Spots" due to manganese deficiency

Apart from this some of the external symptoms incited by fungi on seed surface are given in table 7.1;

Table 7.1 External symptoms produced on seed surface by some seedborne fungal pathogens

Host	Symptoms	Pathogen
<i>Allium cepa</i>	Seed rot Shrunken seeds	<i>Alternaria porri</i> * <i>Peronospora destructor</i>
<i>Cicer arietinum</i>	Reduction in seed size	<i>Ascochyta rabeii</i>
<i>Coriandrum sativum</i>	Hypertrophy	<i>Protomyces macrospores</i>
Crucifers	Reduction in seed size and seed rot	<i>Phoma llingum</i>
<i>Daucus carota</i>	Seed rot	<i>Alternaria radicina</i>
<i>Dolichos lablab</i>	Red discolouration around micropyle (red nose) Brown spot	<i>Stemphyllum botryosum</i> <i>Colletotrichum lindemuthlanum</i>
<i>Glycine max</i>	Purple stain Crust of oospores	<i>Cercospora kikuchii</i> <i>Peronospora manchurica</i>
<i>Hordeum vulgare</i>	Fungus coating and reduction in seed size	<i>Drechslera teres</i>

Host	Symptoms	Pathogen
<i>Linum usitatissimum</i>	Reduction in seed size	<i>Septoria linicola</i>
<i>Oryza sativa</i>	Fungus coating Pink stain	<i>Drechslera oryzae</i> <i>Tricochoniella padwickii</i>
<i>Phaseolus aureus</i>	Black, brown or grey necrotic lesions	<i>Fusarium equiseti</i> <i>Fusarium semitectum</i>
<i>Pisum sativum</i>	Brown spot Seed rot	<i>Ascochyta pisi</i> <i>Mycosphaerella pinodes</i>
<i>Sorghum vulgare</i>	Shrunken seeds	* <i>Sclerospora sorghi</i>
<i>Triticum vulgare</i>	Fungus coating Shrivelled seeds Shrunken seeds	<i>Fusarium graminearum</i> <i>Septoria nodorum</i> * <i>Puccinia graminis</i>
<i>Zea mays</i>	White streak radiating from embryo Black streak radiating from embryo Shrunken seeds Seed rot	<i>Fusarium moniliforme</i> <i>Drechslera maydis</i> * <i>Sclerospora philippinensis</i> <i>Fusarium graminearum</i>

*Not seedborne

7.1.2 Washing Test

Washing test is being done to see the presence of seed mycoflora on seed surface as it provides the information about the presence of the various pathogenic and non-pathogenic fungal species in the field when the crop matured. Here 50g of seed is taken in a conical flask with 50ml of water and shaken for ten minutes on a mechanical shaker. The suspension is examined either as such or the suspended spores are concentrated by centrifuging at 3000 rpm for 15 minutes. The supernatant is discarded and the pellet deposited on the side walls of the centrifuge tube is re-suspended in 0.2ml of lactophenol (a mixture of lactic acid, phenol, water and glycerol in the ratio of 1:1:1:2). This suspension is then examined under the microscope for the presence of spores, conidia and other fructifications. At a magnification of 250-400x, the spores are counted within the over all squares of the haemocytometer 10 times. If spores are found only outside the square, it is counted as less than 1 instead of zero. Rice (1939) suggested the use of haemocytometer for the semi-quantitative estimation, where the spore load per gramme of seed can be calculated with the help of the following formula:

$$\text{Number of spores per gramme of seed} = \frac{\text{Av. no. of spores per counting volume} \times \text{volume of suspension in mm}^3}{\text{Counting volume in mm}^3 \times \text{weight of working sample in gramme}}$$

7.1.3 Incubation Method

The incubation method is again divided into two i.e. blotter method and agar plate method. Both these methods are recommended by International Seed Testing Association (ISTA) and is suitable for pathogens producing mycelium, spores or other fruiting bodies.

7.1.3.1 Blotter method

Blotter method was developed by Lucie Doyer (1938). She was the first seed pathologist in the world who started with an official seed health testing programme at the Government Seed Testing Station in Wageningen, The Netherlands in 1919. Doyer's favourite technique for cereal seeds was to grow them on damp blotters in shallow zink trays. She used to examine the seedlings for disease symptoms under an ordinary binocular microscope as the stereo binocular microscope or a zoom lense were not developed at that time. For most of the fungi an incubation period of 5 to 7 days was found sufficient to develop in their own way so that it was possible to identify them. Dr. C. Wehlburg succeeded Lucie Doyer and in October, 1948 de Tempe joined the Government Seed Testing Station, in Wageningen as Wehlburg's successor. In 1977 de Tempe retired at the age of 63 and was succeeded by Dr.C.J.Langerak. The blotter method was later included in International Seed Testing Association Rules of 1966 for routine seed health testing. ISTA has prescribed following five conditions for routine seed health testing by blotter method:

- (i) Four hundred seeds are to be tested
- (ii) Twenty five seeds (if the seeds are small) and ten seeds (if the seeds are large) are to be plated equidistantly in each plate.
- (iii) The plates are to be incubated at 20°C for seven days.
- (iv) There should be an alternate cycle of light and darkness i.e. 12h light and 12h darkness.
- (v) The distance between light source and the plates should be 40cms

Usually 400 seeds are sown on three layers of blotters moistened with tap water in plastic Petri plates. If the seeds are small in size (like wheat, rice, mustared

etc.) 25 seeds per plate and if they are large in size (like maize, beans etc.) 10 seeds per plate are sown equidistantly as shown in figure on page 326. The plates are incubated at 20°C under diurnal cycle of 12h darkness and 12h light. The plates are placed in a single layer and not stacked. Normally, the NUV (near ultra violet) light should be used- which are also known as black light tubes- but in most of the South-East Asian countries black light tubes are not available and hence normal day light tubes can be used with same efficiency. The distance between the light source and the plates should be 40cms. After incubation the plates are examined under stereobinocular microscope for the presence of fungi like *Alternaria*, *Curvularia*, *Drechslera*, *Fusarium* and *Phoma* etc.

Blotter test is a combination of *in vitro* and *in vivo* principles of investigation. The stereobinocular microscope enables the observation of fungi as developed on their host, *in situ*, undisturbed and in a condition of natural growth. There are two modifications of the blotter method:

7.1.3.1.1 2, 4-D method It is a modification of blotter method and so instead of water the blotters are soaked in 0.2 per cent solution of 2, 4-dichlorophenoxy acetic acid (2, 4-D). Incubation and other conditions remains the same as in normal blotter method. Since 2,4-D retards seed germination and seedling growth, facilitating the examination quick and easy. Neergaard and Saad (1962) observed that *Pyricularia oryzae* in rice gave a higher count by this method. In 1973 the Plant Disease Committee of ISTA tried another growth retardant-Coumarine, in its comparative seed health testing programme for the detection of *Pyricularia oryzae*, *Drechslera oryzae* and *Alternaria padwickii* in rice. However, it gave a lower count as compared to standard blotter method and the compound has a bleaching effect on these pathogens. It even affected the morphology of the pathogens.

7.1.3.1.2 Deep freeze method This is another modification of blotter method which was developed by Dr. Theunis Limonard in 1966. In this method the seeds are plated as in standard blotter method but the plates are first incubated at 20°C for 48h to induce germination and then transferred to -20°C in a deep freezer for 24h and again brought back to 20°C under NUV or normal day light tubes for rest of the incubation period of 3-5 days. Fungi like *Fusarium* and *Septoria* in cereals, *Phoma* in sugar beet and *Pyrenophora graminia* and *P. teres* in barley can be detected by these methods. This method is superior to 2,4-D blotter method as in 2,4-D method often the spores get swollen or there is a change in their shape. Rennie and Tomlin (1984) suggested that for detection of *Septoria nodorum* the seeds may be incubated on moist blotter for 24h followed by 24h at -20°C and then incubated at 20°C for rest of the 5 days.

7.1.3.2. Agar plate method

This method is used for identification of fungi associated with seeds based on growth and colony characteristics on a nutrient medium. This method was used for routine seed health testing of flax in Ulster without pre-treatment of seeds and so it is popularly known as Ulster method as well (Muskett and Melone, 1941).

- (a) In this method generally Potato Dextrose Agar (PDA) is used as a substrate. About 200g of peeled and sliced potato is boiled for 15mts in 500ml of water. The contents are then filtered through a musline cloth. The potato slices are thrown off and the starch extract is kept separately. Now 20g of agar powder is boiled in another 500ml of water till it is completely dissolved in water. Then the starch extract and the agar solution is mixed in an one litre flask or beaker. Finally 20g of dextrose is added and the volume is made up to 1000ml. This PDA is divided in to four 250ml conical flasks and plugged with non-absorbent cotton plug and autoclaved at 20 lbs. psi for 20mts.
- (b) The glass Petri plates should be washed properly, followed by sterilization which can be done either in hot air oven at 160°C for 8h or they may be wrapped in butter paper and then autoclaved at 20lbs. psi for 20mts.
- (c) Keep the laminar flow ON for 15mts before pouring the media into the plates. When the temperature of the media comes down to around 50°C, carefully lift the lid of the previously sterilized Petri plate and pour about 20ml of the PDA into it. Stack the plates one over the other and allow the PDA to solidify. These plates should be used after 24h. Plates showing any contamination should be discarded.
- (d) Pre-treatment of seeds is essential in agar plate method. The seeds should be treated with 0.1 per cent solution of mercuric chloride ($HgCl_2$) followed by washing with distilled or sterile water 2-3 times to remove the traces of mercury. The seeds are picked up with an sterile forceps and placed on PDA.
- (e) Normally, 10 seeds are placed equidistantly but if the seeds are of bigger size ,5 seeds per plate may be sown. If the studies are to be conducted for some storage fungi like *Aspergillus* or *Penicillium* then 180g of sodium chloride per liter of PDA is to be added. The plates are incubated at 20°C for five days under alternate cycle of light and darkness. In this method the identification is based on colony characters only which may be, at times, misleading. There are two modifications of the Agar Plate method:

7.1.3.2.1 Hold-fast method This is a modification of agar plate method which was developed by Mangan (1971) and is specific for the detection of *Phoma betae* a common pathogen in sugar beet seeds. Here instead of PDA 1.2 per cent water agar is prepared. The medium and the glass plates are sterilized as in agar plate method. The water agar is poured in the sterilized glass plates and are allowed to solidify. The pre-treated seeds of sugar beet are placed on the medium and incubated at 20°C for 5-7 days. After incubation hold fast like structures (swollen hyphal tips) develop which can be seen from the back side of the plate under the stereo binocular microscope. Development of hold fast like structures indicates the presence of the pathogen in the seeds.

7.1.3.2.2 Guaiacol method This is another modification of agar plate method which was developed by Kulik (1973) and is specific for the detection of *Pyricularia oryzae* in rice causing blast disease of rice. In this method 0.124g of Guaiacol and 0.146g of Coumarine is dissolved in 20ml of distilled water, which is then added to 980ml of distilled water. After adding 5g of agar powder the medium is autoclaved at 20lbs. psi for 20mts. As soon as the medium cools down to about 50°C, 0.5g of Streptomycin is added to it. Now the medium is poured into sterilized glass Petri plated aseptically. The plates are stacked one over the other and the medium is allowed to solidify. Rice seeds pre-treated with 0.1 per cent solution of mercuric chloride and then washed 2-3 times with distilled water to remove the traces of mercury are then sown on the medium and incubated for 4 days. A pink coloured halo develops around the infected seeds indicate the presence of the pathogen in the seed and can be seen by naked eye. The number of infected seeds are counted and the result is reported in percentage

7.1.4 Sodium hydroxide seed soak method

7.1.4.1 Detection of Karnal bunt and rice bunt

The NaOH seed soak method was first developed by Agarwal and Srivastava (1981) for the detection of Karnal bunt in wheat caused by *Neovossia indica* and rice bunt in rice caused by *Neovossia horrida*. In this method the seeds are soaked in 0.2 per cent solution of NaOH for 24h at 25° or 30°C. The next day the solution is decanted and the seeds are thoroughly washed in tap water. Then the seeds are spread over a blotter paper so that excess of water is absorbed by the blotter. Now the seeds are examined visually. The rice seeds showing dull black or shiny jet black discoloration may contain bunt infection of *Neovossia horrida*. This may be confirmed by rupturing each discoloured seed in a drop of water with a fine needle for the release of bunt spores. A stream of teliospores of *N. horrida* is

released from shiny jet black infected seeds. The insipient infection of Karnal bunt can also be detected in the same way. The NaOH treatment clears the seed surface and increases the colour contrast between the infected and healthy seeds, which helps in easier, quicker and accurate estimation of the infection. This method can be used for both treated and untreated seed lots. The method is less time consuming as compared to examination of dry seeds (Agarwal and Mathur, 1992; Agarwal, 1993). Satyanarayana *et al.*, (1991) and Chahal and Panru (1997) also found this method more sensitive for the detection of rice bunt.

7.1.4.2 Whole embryo count method

Maddox (1896) for the first time observed the mycelium of *Ustilago segetum var tritici* causing the loose smut in the embryos of wheat seeds. Westmann (1942) observed the mycelium of loose smut in the microtome sections of wheat seeds. According to Popp (1958) the fungus stains deep blue, the cell wall of the host stain light blue and the cell contents of the host do not take any stain. Naumova (1957) reported that loose smut infected seeds exhibit blue fluorescence under UV light. Fialkovaskoya (1963) found that seed coat of seeds with heavy infection of loose smut lose lusture and become dull. However, all these methods were unreliable and so could not be used for routine seed health testing for loose smut. The embryo count method was first applied by Skvortzor (1937) for the detection of loose smut fungus in wheat. Since then numerous modifications in this method were done. Fifteen hundred wheat seeds are soaked for 20h at 20°C in 5 per cent solution of NaOH or KOH containing 0.15g of Trypan blue per litre. The soaked seeds are then transferred to a set of sieves arranged in an ascending order of 10, 20 and 50 mesh size so that the 10 mesh size sieve is at the top. A stream of hot water at 60°C is passed over the soaked seeds to separate the embryos from the chaff. Most of the embryos are collected in the middle sieve, while the chaff remains on the top sieve. The embryos thus separated are once again washed with water to remove the traces of NaOH or KOH and later transferred to ethyl alcohol for two minutes for dehydration. The dehydrated embryos are transferred to a funnel, the stem of which is attached to a rubber tube with a stop cock. A 3:1 mixture of lactophenol and water is added. The embryos float and the chaff sinks which can be drained off easily into a beaker. This process is repeated 3-4 times till the embryos are free from chaff. The clean embryos are then gently heated avoiding vigorous boiling in a fresh solution of lactophenol to remove the excess of stain, if any. Once the lactophenol cools down, the embryos are ready for examination. The embryos are examined at 12 or 25X magnification of stereobinocular microscope. The

Infected embryos have bluish thread like mycelium (with uniform thickness and swellings) which may be present in scutellum, plumule bud or the whole embryo.

7.2 DETECTION OF VIRUSES

Like fungal pathogens viruses also produce some visible symptoms on seed surface. It is rather difficult to describe all the symptoms produced by viruses on seed surface as many have not been even reported, yet an attempt has been made to list some of them in the table given below:

Table 7.2 Morphological seed abnormalities correlated with infection of some seed transmitted viruses

<i>Plant species</i>	<i>Seed abnormality</i>	<i>Probable responsible virus</i>
<i>Allium cepa</i>	Reduced seed size	Onion yellow dwarf
<i>Arachis hypogaea</i>	Small size, discoloured seed-coat	Peanut mottle
<i>Cucumis melo</i>	Poorly filled, deformed seeds	Squash mosaic
<i>Cucurbita pepo</i>	Poorly filled, deformed seeds	Squash mosaic
<i>Glycine max</i>	Discoloured seed coat	Soybean stunt
<i>Hordeum vulgare</i>	Small size, shrivelled seed	Barley stripe mosaic
<i>Lactuca sativa</i>	Light weight	Lactuca mosaic
<i>Lupinus luteus</i>	Large or sharp edged	Bean yellow mosaic (Lupid narrow leaf strain)
<i>Lupinus luteus</i>	Large size	Cucumber mosaic
<i>Lycopersicon esculentum</i>	Necrotic, blackened	Tobacco mosaic
<i>Vigna radiata</i>	Wrinkled, shrivelled	Mungbean mosaic
<i>Pisum sativum</i>	Wrinkled, greenish grey seed coat	Pea early browning
<i>Pisum sativum</i>	Cracked but healed seed coat, small size or abnormal shape	Pea seedborne mosaic virus
<i>Vicia faba</i>	Peripheral brown necrosis of seed coat	Broad bean stain
<i>Vicia faba</i>	Discoloured seed coat	Bean yellow mosaic
<i>Vigna unguiculata</i>	Shrunken, shrivelled	Cowpea aphid borne mosaic

7.2.1 Enzyme linked Immunosorbent Assay

Voller *et al* (1974) introduced the microplate method of ELISA, which has subsequently been used, for the diagnosis of wide variety of low concentrations of virus and specific antibody first by Voller *et al* (1976) and later by Clark and Adams (1977).

7.2.1.1 DAS-ELISA

The DAS-ELISA is the most widely used procedure for the detection of viruses was given by Clarks and Adams(1977). In DAS-ELISA the virus is trapped by antiviral antibody and the same is detected using enzyme linked virus specific antibody. The procedure is quite elaborate and essentially require following articles for conducting the test:

- (i) Microtitre plates
- (ii) Multichannel volume adjustable pipettes (20-200 μ l)
- (iii) Plate washer
- (iv) ELISA reader
- (v) pH meter
- (vi) Disposable tips for the pipette
- (vii) Glasswares

Apart from this following buffers and chemicals are also needed:

- (i) Coating buffer (pH 7.4)
- (ii) Washing buffer (pH 7.4)
- (iii) Extraction buffer (pH 7.4)
- (iv) Conjugate buffer (pH 7.4)
- (v) Substrate buffer (pH 9.8)
- (vi) Purified γ -globulin
- (vii) Alkaline phosphatase labeled γ -globulin-anti rabbit IgG in goat(SIGMA)
- (viii) p-nitrophenyl phosphate (SIGMA)

7.2.1.1.1 Protocol of DAS-ELISA

- Add 20 μ l of purified IgG in 20ml of coating buffer. Add 200 μ l of diluted IgG in each well.
- The plates are sealed with plate sealer and incubated at 37°C for 4h.

- Wash the plates 3 times, each for a duration of 3 minutes, with washing buffer. Tap the plates on a soft pad to remove the buffer.
- Add 200 μ l of test sample prepared in extraction buffer in each well. Incubate the plates at 4-6°C overnight.
- Wash the plates 3 times, each for a duration of 3 minutes with washing buffer. Tap the plates on a soft pad to remove the buffer.
- Add 200 μ l of enzyme conjugate diluted 1000 fold in conjugate buffer in each well and incubate at 37°C for one hour.
- Wash the plates 3 times, each for a duration of 3 minutes with washing buffer. Tap the plates on a soft pad to remove the buffer.
- Add p-nitrophenyle phosphate (PNP) @ 1 mg / ml of substrate buffer. Add 200 μ l of this in each well and incubate at room temperature in dark for 30 minutes.
- Read the plates with the help of ELISA reader at 405nm for OD values.

7.2.1.1.1.1 Flow Chart for DAS-ELISA

Add 200 μ l of coating buffer containing purified IgG @ 20 μ l/20ml of coating buffer



Incubate for 4 hours at 37°C



Wash the plates with washing buffer
(3 times, each with 3mts. duration)



Add 200 μ l of test sample in each well
(one part of sap in ten parts of extraction buffer)



Incubate the plates overnight at 6°C

Detection of Seedborne Pathogens

Incubate the plates for 2 hours at 37°C



Wash the plates with washing buffer
(3 times, each with 3 mts. duration)



Add 200 µl p-nitrophenyl phosphate in
substrate buffer @ 1 mg / ml of substrate buffer



Incubate the plates at room
temperature for 30 minutes in dark



Read the plates with ELISA reader at 405 nm.

7.2.1.1.2 Preparation of Buffers

Coating buffer (pH 7.4)

NaCO ₃	4.29 g
NaHCO ₃	2.93 g
Distilled water	1000 ml

Washing buffer (pH 7.4)

NaCl	8 g
KH ₂ PO ₄	0.2 g
Na ₂ HPO ₄	2.9 g
KCl	0.2 g
Tween	0.5 ml
Distilled water	1000 ml

Bovine albumin serum (BSA)	2 g
PBS	1000 ml

Substrate buffer (ph 9.8)

Diethanolamine	97 ml
Na ₂ S ₂ O ₃	0.2 g
Distilled water	1000 ml

7.3 DETECTION OF BACTERIAL PATHOGEN

For the detection of various bacterial pathogens inoculation technique could be adopted. In this technique the indicator or susceptible host plant is inoculated with the seed extract and the symptoms are observed. The infectivity test is simple and reliable but it can only tell that a particular seed lot is carrying the bacteria or not but does not provide the per cent seed infection in a seed lot.

7.3.1 Detection of *Pseudomonas savastanoi* pv. *glycinea*

Kennedy (1969) macerated 10g of dry seeds of soybean in a blender for one minute and then the macerated seeds were put in 50ml of water in a flask. The flask was then placed on a mechanical shaker for three hours. This solution was used for inoculating the healthy leaves of soybean. The development of symptoms on leaves indicate the presence of the the pathogen in the seed lot.

7.3.2 Detection of *Clavibacter michiganensis* sub sp. *michiganensis*

Toyr (1969) reported that the dry tomato seeds should be first grounded in Ringer's solution. The solution is then filtered and inoculated on the 21days old seedlings of tomato. The symptoms of wilting and stem canker appear on seedlings after 28days. Thus it takes about 50days for the test to be completed.

7.3.3 Detection of *Xanthomonas oryzae* pv. *oryzicola*

The method of inoculating the detached leaf of rice with seed washings was developed by Xie and Maw in 1998. In this method, one per cent water agar

7.3.4 Detection of *Xanthomonas oryzae* pv. *oryzae*

A bacterial suspension of *Xanthomonas oryzae* pv. *oryzae* is prepared by pouring 10ml of sterile water containing 0.85 per cent of sodium chloride (NaCl) in the culture tube having pure culture of the pathogen. The suspension is then adjusted to $10^8 - 10^9$ c.f.u./ml. A sterile scissors dipped in the bacterial suspension is used to cut the 35-40days old, fully expanded leaf of a susceptible variety of rice. The cut leaves are examined after 12-15days for the symptoms of the bacterial blight (CPC, 2001).

7.3.5 Detection of *Xanthomonas axonopodis* pv. *manihotis*

The infected seeds are soaked in sterile water for 2-4 hours at 30°C. Leaves of the 30days old plant are then incubated with the supernatant at 30°C and at high humidity for 15days. The angular water soaked spots appear on the leaf from which the pathogen can be isolated. The method was developed by Persley (1979).

7.4 DETECTION OF NEMATODES:

Like any other fungal, bacterial or viral pathogens nematodes also produce symptoms on various plant parts. Some of the symptoms produced by the nematodes on seeds or other planting material has been given in the table 7.3:

Table 7.3 Symptoms produced by the nematodes on seeds and other planting material

Host/Plant part	Symptoms	Pathogen
Seed		
Wheat	Black seed galls	<i>Anguina tritici</i>
Faba bean	Shrivelled and discoloured seeds, sometimes	

<i>Host/Plant part</i>	<i>Symptoms</i>	<i>Pathogen</i>
Peanut	Shrivelled seeds with dark brown testa	<i>Aphelenchoides arachidis</i>
Peanut	Seed blemished or unsound, pods and hulls dark coloured	<i>Ditylenchus destructor</i>
Bulbs, corms, rhizomes and tubers		
Onion, Garlic	Internal necrosis or rot of sets and cloves, sometimes nematode wool attached	<i>Ditylenchus dipsaci</i>
Ginger	Light brown, watery areas on surface, often in constriction with internal necrosis	<i>Meloidogyne spp.</i>
Ginger	Small, sunken watery lesions on surface	<i>Radopholus similis</i>
Turmeric	Shallow water-soaked brownish lesions and rotting	<i>Radopholus similis</i>
Banana, Abaca plantain (<i>Musa spp.</i>)	Purple to dark brown necrotic lesions throughout cortex of roots and similar lesions in outer tissues of corm	<i>Radopholus similis</i> <i>Pratylenchus coffeae</i> <i>Pratylenchus goodeyi</i>
Banana, Abaca plantain (<i>Musa spp.</i>)	Purple to dark brown necrotic lesions in periphery of cortex of attached roots	<i>Helicotylenchus multicinctus</i>
Taro	Internal red necrotic streaks	<i>Hirschmanniella miticausa</i>
Yam (<i>Dioscorea spp.</i>)	Internal dark brown dry rot in periphery of tuber observed when cut or when epidermis is scraped away. Whole tuber spongy to touch with cracks in surface	<i>Scutellonema bradys</i> <i>Pratylenchus coffeae</i> <i>Radopholus similis</i>
Yam (<i>Dioscorea spp.</i>)	Uneven, knobby tubers; internal necrotic spots	<i>Meloidogyne spp.</i>
Potato	Uneven tubers pitted with dark dry necrotic lesions	<i>Pratylenchus spp.</i>
Potato	Watery tubers, swellings and lesions on surface	<i>Meloidogyne spp.</i>
Potato	Cysts on tubers or in adhering soil	<i>Globodera spp.</i>
Potato	Surface cracking and internal dark brown and peripheral rot	<i>Ditylenchus destructor</i>

Host/Plant part	Symptoms	Pathogen
Seedlings (symptoms after uprooting)		
Vegetables, rice, coffee etc.	Swellings of galls on roots	<i>Meloidogyne spp.</i>
	Discrete swellings or galls on roots	<i>Nacobbus spp.</i>
	Yellow or brown lesions on roots	<i>Pratylenchus spp.</i>
	Distorted root growth with white specks on the surface in older seedlings	<i>Hirschmanniella spp.</i>
Rootstocks		
Grapevine, peach etc.	Galls on roots accompanied with rotting	<i>Meloidogyne spp.</i>
	Brown lesions on root surface	<i>Pratylenchus spp.</i>
Citrus	Irregular, dirty roots with soil adhering to surface	<i>Tylenchus semipenerans</i>

Source: Lal (1995)