Introduction to methods of seed health testing*

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Summary

A survey and discussion are presented of the methods available for routine seed health testing, including crude methods for orientation purposes as well as more refined modern devices. Emphasis is still being laid on the fungal infections and their detection, as these have been studied intensively for quite a long time and form the bulk of the health testing programme in many seed testing stations. The methods for bacterial and virus infections are not so well established and up to now rarely applied in seed testing stations. The present possibilities for these groups of infections are reviewed in separate sections. For further study a list of references has been added.

Résumé

Introduction aux méthodes d'analyse sanitaire des semences.

Cet article présente une vue d'ensemble et une discussion des méthodes utilisables en analyse sanitaire de routine des semences, qu'il s'agisse des méthodes élémentaires à des fins d'information générale ou de techniques modernes plus fines. L'accent est encore mis sur les infections cryptogamiques et sur leur détection, car celles-ci ont été étudiées intensivement depuis un temps assez long et elles représentent l'essentiel du programme d'analyses sanitaires dans de nombreuses stations d'essai de semences. Les méthodes relatives aux infections bactériennes et virales ne sont pas aussi bien définies et, jusqu'à présent, elles sont rarement mises en oeuvre dans les stations d'essai de semences. Les possibilités actuelles relatives à ces derniers groupes d'infections sont passées en revue dans des sections distinctes. Une liste de références a été ajoutée en vue d'études complémentaires.

Zusammenfassung

Einführung in die Methoden der Saatgutsanheitsprüfung**


* Section 1 of the ISTA Handbook on Seed Health Testing.
**Abschnitt 1 des ISTA Handbook on Seed Health Testing.
1. Introduction

Routine health investigation of seeds may be performed for different purposes:
- to detect quarantine infections;
- to study the health condition as one factor among several that determine the field value of the seed;
- to decide whether pesticide treatment of the seed is necessary;
- to assess the prevalence of a seed-borne infection in a survey or the importance of a seed-borne disease in research.

Occasionally health tests will be made for tracing the cause of low germinating capacity or field emergence.

Seed-borne infections are usually more important in starting epiphytopsies than in their effect on seed performance in the germination stage. In the seed testing station seed health should, therefore, not be regarded as subordinate to germinating capacity. It is a plant-pathological matter and should be regarded from a plant-pathological standpoint.

On the other hand, the germination figure is essential for every seed lot but there are many kinds of seeds for which the health condition is not a matter of consequence and not worth investigating. This will also be dependent on climatic conditions in the region where the seed was grown, and often in the region where it will be sown.

The choice of the health testing method to be used will depend not only on the properties of the seed and its infections and the properties of the disease that may be started, but also on the purpose of the test. For a survey, or for deciding whether the seed should be pesticide-treated, an approximation of seed-borne infection is sufficient. As the degree of development of many diseases in the field is not only dependent on the amount of seed-borne infection but often far more on the sowing and growing conditions in the field, an approximate determination may also be quite satisfactory for assessing the field value of the seed. For these purposes a number of methods are available, and it may be sufficient to test only 200 seeds per sample, unless traces of infection can be present and are known to be important.

For plant quarantine work (testing for important seed-borne diseases still absent in a seed-importing country) traces of infection may be dangerous so that more seeds have to be tested and the most sensitive method used. This is also true for infections of which traces in the seed may start a serious disease development in the field, either because of the exceptional spreading capacity of the pathogen (for example many bacteria, viruses and the fungus Colletotrichum lagenarium (Pass.) Ell. & Halst. on Cucurbits), or because disease development is greatest in late development stages of the host, so that even with a low infection percentage in the seed sown there is sufficient time for a strong build-up (Ascochyta pisi Lib. in peas (Pisum sativum L.)).

Though qualitative pathological investigation of seeds may yield valuable results, in most cases quantitative information is required, viz. in seed legislation, certification and for international certificates. With commercial seed lots the financial issues may be
considerable. Then reliable and standardised methods, the results of which can be reproduced for any one sample and compared for different samples, are required.

With most seed health methods percentage figures are produced. Then the quantitative aspect is limited to the percentages of infected seeds whereas the amount of inoculum per seed and its localisation on or in the seed interacting with the sowing conditions will decide whether in normal non-sterile soil this inoculum survives and may cause disease. Infection of individual seeds that are below a certain threshold value may be detected in a laboratory test and yet have no consequences in the field. Other seeds may be seriously infected and develop into abnormal seedlings in the laboratory, but in the field these seedlings will not emerge and not start an epiphytotic in the growing crop whereas plantlets developed from slightly infected seeds may. But if the pathogen is also soil-borne, diseased seedlings which fail to emerge can still cause disease in the future.

It will be clear from the preceding that hard and fast rules for seed health testing cannot be given. A thorough knowledge of each seed-borne infection and of the disease to which it may give rise is a necessity for deciding how many seeds must be tested and by which method. For many combinations of seed and pathogen this knowledge is not available.

In most cases the method used must meet the following requirements:
- the pathogen must be recognisable with ease and certainty;
- the method must provide results that are reproducible for any one sample and comparable for different samples;
- except in cases of quarantine inspection the results should be informative for the possible field performance of the seed, which means that the relation between laboratory test results and disease development in the field should be fairly close;
- the method should be simple, cheap and quick;
- it should be suited for standardisation with respect to international use.

These requirements are difficult to meet. Seed health is a complicated matter, dependent not only on the seed-borne pathogens themselves but also on the presence of other micro-organisms on the seed that may be antagonistic or perhaps synergistic in an incubation test.

Often the level of the laboratory figures is different from, and usually higher than, the percentages of disease appearing after sowing in natural soil. Consequently the level of results of a laboratory method is less important than its correlation with subsequent field observation. Only for important plant quarantine and also for fast-spreading pathogens is this not true. In plant quarantine what ultimately matters is not disease introduction in the crop grown from the seed but disease establishment in the seed-importing country. This is a qualitative aspect. In the case of diseases for which a trace of seed-borne infection is sufficient for serious disease development in the field, the quantitative relation exists theoretically but will soon be obscured in field trials.
A seed may be regarded as a 'biotope', an ecological unit consisting of the seed and its micro-population: fungal and bacterial and other components, both pathogens and saprophytes. This is what makes seed health testing complicated and intriguing, especially when incubation methods (that have the broadest applicability) are used. During incubation various micro-organisms develop, interact, and small changes in testing conditions may cause great differences in results. This restricts the accuracy of those methods and so their value for international use.

Still, even an imperfect method when standardised and used by experienced workers may yield results of great practical value. The present situation is that for many important seed-borne infections various workable methods are available, but the knowledge required for a justified choice of a preferable method and its standardisation is largely lacking. This is mainly so for the incubation methods, and much research on these is still needed.

In the following section a rather detailed but tentative listing of methods for seed health testing is given, with some information on each method added. The listing has to provide sufficient detail to be useful, but this increases the possibility that the information given will soon be obsolete.

In additional information certain pathogens will be occasionally mentioned but this does not imply that the discussed method is preferable for those pathogens.

Seed may have specific physiological disorders, such as marsh spot due to manganese deficiency in large-seeded legumes, or boron deficiency (without direct symptoms — Leggatt, 1948) in peas. These disorders are the consequence but not a starting point of disease development; they are not infectious and can be evaluated as weakness of the seed causing reduced emergence and stand; they are usually more or less reflected in the official germination figure. Still, as physiological disorders are normally regarded as diseases, they have been given a place in the listing.

Finally, it must be emphasised that the utmost care is always required in preparing the working sample, which should reflect the composition of the sample received in the laboratory.

An excellent discussion of seed health testing methods was recently given by Neergaard (1977).

Pesticide treatment is applied against seed-borne infections (disinfectants) and also against the consequences of seed weakness (protectants). True disinfectants are volatile and water-soluble and penetrate into the seed, so that they are very effective against seed-borne infections unless these are too deep-seated. As these chemicals disappear soon after the seed is sown in soil, they are usually unsatisfactory as protectants. True protectants are non-volatile and non-soluble contact fungicides. They are less effective against seed-borne infections but remain in the spermosphere to protect the seedlings during the whole of the emergence period or even longer against pathogen invasion, eventually from their own seedcoats. Systemic fungicides and insecticides may be active against both seed-borne and field-borne invaders. They are taken up by the seedlings and transported in their tissues. Surviving infections in pesticide-treated seeds may require a few extra days for developing in incubation methods.
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2. Provisional list of methods for pathological seed analysis

(NE = naked eye, HL = hand lens, StM = stereoscopic microscope, HPM = high power microscope, FM = fluorescence microscope).

I. Examination of the seed without incubation

A. of the dry seed
1. for impurities such as ergots and other sclerotia, free insects, nematode galls, smut balls, etc.: NE, StM
2. for blemishes, discolorations and malformations indicative of the presence of bacteria or fungi: NE
   a. observed in daylight or ordinary artificial light
   b. observed in ultraviolet light or in other special kinds of light
3. for fungal fructifications such as acervuli, pycnidia, perithecia, resting hyphae on the outside of the seed, or spore masses: NE, StM
4. for insects and insect damage: NE, or mites: StM
   a. in ordinary light
      i. for specific organisms
      ii. for non-specific warehouse pests including mites
      iii. for insect damage caused in the field, whereas the insect itself is not present in the seed sample
   b. with X-rays or other special kinds of radiation
   c. by aural detection
   d. by flotation
5. for pesticide particles or indicative colour: NE, HL, StM

B. after softening, soaking or processing
1. fungal fructifications (see I.A.3.) are often more clearly visible after soaking of the seed in water or in lactophenol: StM
2. fungal spore masses and nematodes: StM, HPM
3. fungal hyphae within the seed can be made visible by soaking and staining: HPM
   a. loose smut fungi in the isolated embryos
   b. downy mildews in the seedcoats
4. for insects within the seeds: NE
5. for deficiency symptoms (see also II.6.): NE

C. the washings from the seed
1. for spores or typical hyphal elements: HPM
2. for eelworms: StM, HPM
3. for bacteria in special preparations: HPM
4. for colour from pesticide treatments, expedited by special preparations: NE

II. Examination of seeds and seedlings with moist chamber methods, viz. after a period on paper or similar substrata

If seedlings are not needed for symptom inspection, germination may be prevented by
adding weedkillers or by deepfreezing the imbibed seeds.

1. for characteristic rots, discolourations and malformations: NE
2. for conspicuous growth of pathogens: NE
3. for fungus spores or fruiting bodies: StM
4. for bacteria, typical spores or hyphal elements in a microscopical preparation, if necessary after staining: HPM
5. for insects and insect damage: caused by specific organisms, warehouse pests, or insects in the field NE
6. for symptoms of physiological disorders: NE
7. for presence of pesticides as indicated by discolouration of seeds or substrata, or by control of mold growth: NE, HL
8. for symptoms of chemical injury: NE
   a. caused by chemical treatment of the seed
   b. caused by weedkiller treatment of the parent plants in the field.

III. Examination after a period in moist media
A. Inert media (sand, chipped or ground brick, vermiculite, perlite, sterilised soil, etc.)
   1. for rots, discolourations and malformations: NE, HL
   2. for chemically induced abnormalities: NE
B. Non-sterile soil
   1. for rots, discolourations and malformations: NE, HL
   2. for chemically induced abnormalities: NE

IV. Examination after incubation on agar media
Germination may be suppressed with weedkillers such as 2,4D
A. for the presence of pathogenic micro-organisms on or surrounding seeds: NE to HPM
   1. after special pretreatments (NaOCl), mainly when non-selective agars are used
   2. without surface-disinfection, often when selective agars are used
   3. seed pretreatment (NaOCl) and agars of high osmotic value are required for studying storage fungi
B. for the presence of fungicides on the seeds as indicated by inhibition of micro-organisms on evenly inoculated agar (bio-assay method): NE

V. Examination of plants or parts thereof in the greenhouse or in the field
A. growing plants from suspected seeds, followed by examination for infections: NE to HPM
B. plants grown in a healthy condition and inoculated with organisms obtained from the suspected seeds: NE to HPM

VI. Special methods for bacteria
A. Serological methods
   1. Double-diffusion technique: NE to StM

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2. Agglutination methods: StM
3. Immunofluorescence technique: FM

B. Phage-plaque method: NE to HPM

VII. Special methods for viruses
A. Electron microscopy
B. Histochemical methods: StM
C. Serological methods
   1. Double diffusion technique: NE to StM
   2. Agglutination methods: StM
   3. Immunofluorescence technique: FM
   4. Enzyme-linked immunosorbent assay

3. Discussion of the methods included in the list

I.A.1. (Impurities in the dry seed)
The seed sample or part of it may be inspected in the dry state for the presence of impurities such as ergots or other sclerotia, free insects, nematode galls, bunt balls and smutted kernels, all of which are separated in seed purity analysis. In health testing they should be identified and recorded. Seed health analysts have to distinguish between ergots (sclerotia of Claviceps spp.) and sclerotia of Sclerotinia, Sclerotium, Botrytis and other genera. The seed species in which they occur may be a help. Where direct distinction of the sclerotia of different fungi is impossible it may be necessary to obtain pure cultures from surface-sterilised sclerotia. The distinction between ergots and Anguina eelworm galls in grasses may require checking in a microscopical preparation (Goodey, 1952). The waxy content of the galls consists entirely of slender nematodes. Bunt (Tilletia spp.) and smut (Ustilago spp. etc.) kernels are easily recognised as such. Identification to genus or species, however, will probably require microscopical investigation. Examples are the sorghum smuts (Leukel, Martin and Lefebvre, 1960) and the common bunt as against the dwarf bunt in wheat (Triticum aestivum L.) (Kietreiber, 1976). When related species have a fundamentally different behaviour, their precise determination is important. So, in countries with a cold winter it may be necessary to distinguish between Tilletia caries (DC.) Tul., cause of common bunt of wheat, and the closely related T. controversa Kühn, which causes dwarf bunt. For the study of storage insects in general, and especially of seed insects, the old publications of Zacher (1927, 1932, 1951) are very helpful. A few days at about 25°C in a gauze-covered bottle may favour the appearance of insects hidden within the seeds.

I.A.2. (Discolourations etc. of the dry seeds)
Discolourations and blemishes due to pathogenic organisms may be conspicuous and identifiable. Attention should also be paid to chaff, straw or to other inert matter in which pathogenic fungi or bacteria may be present.

This method has a restricted value, for it is not possible to determine an exact
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infection percentage by examination of the dry seed. Even in such cases as the dark­brown *Mycosphaerella* spots on green peas (foot rot, blight), or the blackish-brown *Colletotrichum* (anthracnose) lesions on white *Phaseolus* beans, the infection percentages found in an incubation test are usually much higher than those determined in dry seed.

There are some infections such as *Ascochyta pisi* (leaf and pod spot) in peas and *Stemphylium botryosum* Wallr. ('red noses') in *Phaseolus* beans, for which judgment of the dry seed may be facilitated by fluorescence of the spots in near-ultraviolet light (from an incandescent N.U.V. lamp as used in seed laboratories for distinguishing between perennial and annual ryegrasses in a blotter test). Under this lamp *Ascochyta* in peas causes a yellow-green fluorescence, *S. botryosum* in white beans a dull-orange one. This method may be an improvement on the inspection of the dry seed in ordinary light for obtaining a quick impression of the degree of infection of certain pea and bean seed samples, but it is not an accurate health testing method (Anselme and Champion, 1962).

It has been claimed that loose smut (*Ustilago nuda* (Jens.) Rostr.) infection in wheat seed can be found by means of a bluish fluorescence of the infected seeds in ultra-violet light (Naoumova, 1957). Recently Wharton (1967) observed that white dwarf beans (*Phaseolus vulgaris* L.) infected with *Pseudomonas phaseolicola* (Burkholder) Dowson are mainly found in the fluorescent fraction.

I.A.3. (Fungal fructification on the dry seeds)

The stereoscopic microscope is useful for detecting fungal fructifications or resting hyphae on the seeds. This method can be very reliable, for instance when *Septoria* occurs in celery (*Apium graveolens* L.) and parsley (*Petroselinum crispum* Mill.) Nyman ex A. W. Hill) seed. Observation is facilitated by soaking the seeds (I.B.I.) or by incubating them on moist substrate (method II).

Quite often this method has to be supplemented by verifying the identity of the fungus by microscopically examining a spore preparation, for instance because of the possibility of a saprophytic as well as parasitic *Phoma* sp. occurring in celery seed. In addition a viability test may be necessary (Sheridan, 1963). Spore measurements may be made for species determination.

With resting hyphae on the seed surface, causing what without magnification may look like a discolouration, mistakes can be made. For instance confusion is possible between the dangerous 'black patch fungus', *Rhizoctonia leguminicola* Gough & Elliott, on seeds of *Medicago* and *Trifolium* spp. (Elliott, 1952) and *Phoma* or *Cladosporium* infections. A plating test using water agar allows the characteristic black hyphae of *R. leguminicola* to develop.

I.A.4. (Insects or insect damage in the dry seed)

In addition to insects being present among the seeds there may be insects hidden inside them. Some seeds may have holes from which bruchids (*Bruchus* spp., *Bruchidius* spp., *Callosobruchus* spp., *Acanthoscelides* spp., etc.), chalcids (*Megastigmus* spp., *Syno-
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Mapsis spp., Bruchophagus funebris Haw. etc.) or other insects have emerged; in other seeds the insects may still be present, either visible or hidden under the seed coat, as are bruchids and chalcids in legumes and chalcids in certain tree seeds.

The insects may be more or less omnivorous storage pests such as granary weevils (Sitophilus spp., etc.) and moths (Tinea spp., etc.); they may be storage pests of a more restricted feeding range, such as certain bruchids (the bean weevil Acanthoscelides obtectus Say, the Brazil bean weevil Spermophagus subfuscatus Boheman etc.); or they may be restricted in their nutrition to a single host species and then mostly cannot feed on the ripe seeds although they may be found alive in the dry seed (the pea weevil Bruchus pisorum L., the vetch weevils Bruchus hrachia/is Fahraes and others). In these cases they are often difficult to detect in the seed sample. Crushing the seeds or removing the seed coats may reveal insects or injuries caused by them.

Feeding damage without the insects still being present may have been caused by field insects, true storage pests or by specialised seed insects. It may be non-characteristic (for instance grain weevils and the grubs of Laspeyresia spp. in peas) or more or less characteristic (e.g. chalcid flies of coniferous seeds). The damage may be conspicuous, as in the cases mentioned, or it may be difficult to detect but nevertheless important, as is true of the embryoless seeds of Umbellifers caused by the bug Lygus campesiris L. (Plemion and Olson, 1950).

A stereoscopic microscope is necessary for proving the presence of mites (Tyroglyphus spp. and others) in musty smelling or dusty samples. Then the dust has to be screened off and examined at low magnification for the presence of live or dead mites and their eggs.

When inspecting stored grain for insects X-rays may be helpful (Dupont Inc., 1953; Kamra, 1964). This technique may be of use in the quarantine station, for which reason it has been included in the schedule as method I.A.4.b. Moreover, aural examination of stored seeds by means of a sound amplifier is practised for foods and fodders in warehouses. Wolfe, Milner and Shellenberger, 1955; however, is scarcely practicable for seed sample infection in the laboratory. The same may be said of the flotation methods, in which fluids of different specific weights are used for separating sound and infested seeds or for collecting insect remains from crushed seeds (White, 1957; Piltz, 1955).

I.A.5. (Pesticides on the dry seed)

Pesticides present on the seed are often visible to the naked eye; sometimes a stereoscopic microscope may help. Liquid fungicides usually contain a distinctive dye. However, I.A.5. is only a crude method of which little needs to be said. A refinement is available in I.C.4.

Seed samples treated with pesticides usually have a sufficiently distinctive smell and appearance indicating the necessity of taking due precautions in handling the seed. Unfortunately the same colours that are used as a warning in commercial pesticide formulations are sometimes used for denaturation of grain which is not to be used for human consumption. In case of doubt special fungicide tests have to be made (II.7., IV.B.).
The principle of group I.B. (softening, soaking or decomposing to make the signs of disease and injury better visible) has already been mentioned in connection with inspection for fungal fructifications.

I.B.1. (Fungal fructifications on the soaked seeds)
When infected seeds of celery or parsley are placed in water or lactophenol, the swollen pycnidia readily reveal the presence of *Septoria* infections. A viability test may be desirable (Maude, 1963).

I.B.2. (Spore masses and nematodes from the seeds in water)
A number of seeds are placed in water and examined under the microscope for fungus spores or for eelworms. The method is useful for *Guignardia fulvida* Sanderson in flax (*Linum usitatissimum* L.) of which the small hyaline cylindrical spores are visible in the swelling slime layer, for the above-mentioned *Septoria* infections, and for *Gloeotinia temulentana* (Prill. & Delacr.) Wilson, Noble & Gray in ryegrass of which the honeydew spores make the water cloudy if single seeds are placed in drops of water.

For certain cases 100 × magnification is quite sufficient, as it is not the spores themselves but the gross appearance which is typical (Wallen and Skolko, 1951). An additional viability test may be necessary.

Also the content of *Anguina* galls may be investigated in water (Doyer, 1938, plate X).

I.B.3. (Internal fungus infections after processing the seeds)
Sub-method a refers to the techniques of demonstrating internal smut fungus infections, that have been developed in Europe and in Canada (Morton, 1961; Popp, 1951; Russel, 1950; Simmonds, 1946; Wöstmann, 1942; Yablakova, 1944).

The loose smut fungus (*Ustilago nuda* in barley (*Hordeum vulgare* L.) and wheat) is present as hyphal strands within the embryo. The effective infection may also be investigated by testing growing points (Morton, 1961). Wöstmann (1941) published a method in which the seeds are swollen, sectioned, stained with fuchsin, and investigated in ultraviolet light for fluorescent mycelium (50-300 × magnification).

The Canadian methods amount to macerating the cereal seeds in sodium hydroxide solution, isolating the embryos, clearing these, and finally eventually staining and, if necessary, crushing them. The characteristic hyphae are easily recognised in the embryos with low magnification (about 20 × ) and hence the percentage of infected seeds can be reported.

A similar technique can be used to demonstrate, or to determine quantitatively, the presence of *Ustilago avenae* (Pers.) Rostr. in grains of oats (*Avena sativa* L.). The hyphae of this smut fungus become established in the seed coats following floral infection.

By removing the seeds from the enclosing glumes after moist incubation, taking off the seed coats and staining these in cottonblue, the hyphae are evident at a magnification of about 450 × (Zade, 1940).
Advantages of the embryo-tests are quick dependable results and no influence of environment. False loose smut of barley (Ustilago niger Tapke), which contrary to U. nuda may be controlled with mercurials, is not included in the resulting percentage. False loose smut of barley (Ustilago niger Tapke), which contrary to U. nuda may be controlled with mercurials, is not included in the resulting percentage. False loose smut of barley (Ustilago niger Tapke), which contrary to U. nuda may be controlled with mercurials, is not included in the resulting percentage. False loose smut of barley (Ustilago niger Tapke), which contrary to U. nuda may be controlled with mercurials, is not included in the resulting percentage. False loose smut of barley (Ustilago niger Tapke), which contrary to U. nuda may be controlled with mercurials, is not included in the resulting percentage.

Sub-method b consists of killing and fixing the seeds in a suitable solution, sectioning, clearing and staining preparatory to examination under high power magnification (approx. 450 x). Since the hyphae of downy mildew fungi are commonly present in the intercellular spaces of the seed coats, simply macerating the seed coats, clearing and staining with cotton blue will suffice to indicate their presence. A specific identification cannot be accomplished but the non-septate hyphae in seeds of peas and Amaranthus spp. strongly suggests a species of Cystopus or Peronospora. P. manshurica (Naum.) Syd. enroots soybean (Glycine max (L.) Merr. seeds. Oospores if present will fix the genus and possibly the species (Melhus, 1931).

I.B.4. (Insects within the softened or soaked seeds)

Insects within seeds can be detected by soaking the seeds with sodium hydroxide followed by inspection with a stereoscopic microscope (Piltz, 1955).

Simply swelling the seeds in water facilitates opening them for internal inspection.

I.B.5. (Deficiency symptoms in the softened seeds)

The internal necrosis of pea, bean and Vicia bean seeds, caused by manganese deficiency, is present within the dry seed but can be detected more readily when the seeds are opened after swelling. Normally, however, this will be studied together with other abnormalities at the end of an incubation test (II. A. or II.B.), when growing point injury can also be evaluated.

Group I.C., examination of the washings from the seeds, is of great value for studying certain disease conditions.

I.C.1. (Fungal elements in washings from the seed)

This technique has been of great practical importance for a long time (Heald, 1921). After the original publications concerning bunt of wheat this method was introduced into several European seed testing stations. New modifications have since been developed (Cherewick, 1947; Leblond, 1948; Keding, 1952; Kietreiber, 1952, 1976).

Bunt of wheat (Tilletia spp.) is transmitted mainly by means of characteristic dark spores adhering to the seed surface (Heald, 1921). These spores are shaken off with water and collected by means of filtering. Then the filter itself is cleared and examined (Kietreiber, 1976).

For Tilletia spp., a magnification of 100 x is sufficient for counting, but for species identification a higher magnification is needed. The dwarf bunt fungus, T. controversa, has already been mentioned in I.A.1. of this section (Fischer, 1952; Warmbrunn, 1952).

It is necessary to distinguish between T. caries and T. foetida (Wallr.) Liro. on the one hand and T. controversa on the other. One reason is that the dwarf bunt fungus is only locally present and may be spread and become soil-borne by means of seed-borne spores. Also it is more difficult to control than the other two species. T. caries and T.
contraversa can be distinguished in a preparation in dilute Indian ink, this suspension of carbon particles revealing the thick slime layer surrounding the dwarf bunt spores. Also Shear’s medium can be used but Kietreiber (1976) recently developed a new method.

Technique I.C.1. can be used for the detection of every fungus with characteristic spores or hyphae present in sufficient numbers on the outside of seeds and not themselves obscured by too much dirt, even when these surface-borne spores are not important for transmission of the disease. In addition to Tilletia spp. the surface-borne covered smuts may be thought of (Davis, 1935). The method is less reliable in the case of Ustilago avenae and U. hordei (Pers.) Lagerh., in which the spores partly penetrate between the glumes and the caryopsis (Gassner, 1953). Yet probably only these may infect the seedlings. For Tilletia spp. a magnification of ×100 is satisfactory; for the small-spored Ustilago spp. a larger magnification is necessary. The method has also been used for Gloeotinia temulenta in ryegrass (Hardison, 1948) and for Cereospora beticola Sacc. in beets (Wenzl, 1959). In these cases the fungus spores may have died off but are accepted as proof of a proportional mycelial infection of the seed.

In Canada method I.C.1. has been used in large-scale research programs (Greaney, 1944; Leblond, 1948; Simard and Ludwig, 1948). That it should be used with caution is made clear by the example of Drechslera avenae (Eidam) Scharig, in which most of the infected seeds did not carry spores (Turner and Millard, 1931).

To conclude we may remark that this method I.C.1. is closely related to I.B.2. (examination of the seeds in water). The latter is restricted to cases in which the spores themselves are not very characteristic, but the infection picture including the seed is. Method I.B.2. relies on the presence of great numbers of non-diagnostic spores; method I.C.1. is only satisfactory if definitely identifiable spores are present.

I.C.2. (Nematodes in washings from the seed)

This method is important for some prevalent seed-borne pathogens. The stem eelworm, Ditylenchus dipsaci (Kühn) Filipjev, may be present in a desiccated state on the outside of as well as within the seeds of clover (Goodey, 1950), onion (Allium cepa L.) Vicia beans and other species. By placing a certain quantity of seed on filter paper in a hopper of which the outlet has been closed with a tap, and putting the hopper underneath a fine water spray, the eelworms are provided with moisture and air, start moving and sink through the filter paper until just above the tap whereas surplus water flows off over the border of the hopper. Next day the few drops of water which may contain eelworms are drawn off in a watch glass and inspected for the presence of this nematode (for its characteristics see Goodey, 1963). For clover and onion seed the method appears to be workable, but its results may be very irregular probably owing to most of the eelworms originating from the very few pieces of inert matter in the sample (Ubels, 1952). High power magnification may be needed for species determination.

I.C.3. (Bacteria in preparations cultured from the seed)

This refers to the partial determination of bacterial species, for instance certain plant
parasites belonging to the genus *Corynebacterium*, which give a positive reaction to the Gram stain and have a typical coryneform shape whereas most other plant-pathogenic bacteria are 'Gram-negative' (Dowson, 1957). For full identification the organism should be isolated and cultured.

1.C.4. (Pesticide colour in washings from the seed)
This is a good method for proving the presence of powder and liquid seed treatments. Direct examination (1.A.5.) can detect fungicide or insecticide particles on the seed, but liquid pesticides on dark seeds are not always noticeable. In many cases simply shaking the seed with water, alcohol, carbontetrachloride or other solvents will reveal the presence of pesticide by a colour change of the solvent. This may also be important in the seed testing station for protecting the health of the workers.

Where more detailed information is necessary certain reactions are available for distinguishing between various groups of fungicides and insecticides, even in the small quantities of these substances present in a seed sample. For mercurials a colour reaction with dithizon solution (diphenylthio-carbazon) is known, resulting in a yellow colour if mercury is present. Also for different groups of protectant fungicides specific tests have been developed (Burchfield and McKew, 1948; Domar, Fredga and Lindberholm, 1949), and the same is true for the insecticides DDT and BHC (Carter, 1947; Schechter and Hornstein, 1952). However, this is outside the scope of activities of a seed testing station.

The methods of groups II to IV are the very important incubation methods. They have the broadest applicability, the blotter test being suited for all infections that appear themselves or cause characteristic symptoms in the seedling stage, and the agar test being suited for all infections that develop quickly and characteristically on nutrition agars. Whereas the methods discussed earlier are subject to a statistical error and sometimes an error in judgment, but more often are unsatisfactory because not all infected seeds show the symptoms mentioned, the incubation methods are subject to an additional source of error, viz. the consequences of small changes in incubation conditions. These can be very important for a pathogen which is part of a complicated ecological system.

When blotter and agar methods are compared for the same infection, the agar method will often produce higher figures. The main reason for this is that in the agar method pathogens develop saprophytically on a rich medium whereas in the blotter method nutrition is not always plentiful and also the substrate, either living host tissues or dead seedcoat layers or glumes, have a certain degree of resistance against fungus invasion. This resistance can best be broken by killing the host tissues by means of deep-freezing, so that much nutrition becomes available.

Antagonism plays an important role both in the agar and in the blotter test. Even when low-pH-agars such as potato-dextrose or malt agar are used, bacteria may grow from the seeds and prevent fungus colonies from appearing or from developing characteristically. Antibiotics can be added to the agar for suppressing bacteria. In the blotter test saprophytic seed-borne bacteria may be active that are antagonistic to the patho-
genic fungi which must be detected. A substrate with a high moisture level stimulates these bacteria, particularly if the seeds are small or slimy. An increased development of bacteria will hamper the fungi, which results in decreased infection percentages being found for the latter. By adding antibiotics such as terramycin or streptomycin, or by using rather dry blotters, the bacteria may be controlled.

Interfungal antagonism is more difficult to control selectively. A copious development of saprophytic fungi may prevent growth or detection of pathogenic fungi in agar tests, and also certain profusely growing pathogenic fungi may prevent observation of other pathogens of a more modest growth habit. For this reason naked-eye inspection of the agar plates is often not sufficient. Also in blotter tests the prevalence of saprophytic fungi may hinder observation of pathogens, so that slightly lower percentages will be found for the latter.

In the agar method superficial disinfection of the seeds (commonly indicated as 'pretreatment') with sodium hypochlorite is often a necessity in the routine investigation of seeds when plated on non-selective agars. Particularly coarse agricultural seeds such as cereals, large-seeded legumes or beets, and fine 'seeds' that have grown enclosed in a fruit structure (Compositae, Umbelliferae, spinach (Spinacia oleracea L.) etc.) require such as a superficial cleaning.

If more or less superficial pathogenic infections are present these will also be reduced by the disinfection; pretreatment. This may decrease the value of the results in cases in which also these slight or superficial infections can cause disease when the seed is sown in natural soil under normal conditions, but it may increase their value should the minor infections be harmless in field sowing. This has to be studied for each infection for which the agar method will be used.

It may happen that in the agar test the disinfection pretreatment scarcely reduces the pathogenic infection, as especially in the absence of saprophytes a trace of pathogen surviving will be sufficient to start a colony. When the same pretreatment is used before testing in blotter medium (which is rarely necessary) a certain minimum amount of surviving inoculum per seed may be required for causing disease. The same is still more true when the seed is sown in natural soil, in which also soil-borne antagonistic micro-organisms may prevent seed-borne pathogens from causing disease symptoms in the host.

In certain cases interfungal antagonism can be largely eliminated in agar tests by using specific agars. Even then a 'chlorine' pretreatment may be useful for reducing the infection to an agriculturally justified level. Occasionally antagonism can be reduced in blotter tests by special pretreatments such as a dry heat treatment of the seed in the case of Drechslera infections of cereals (Malone, 1962; Limonard, 1968).

In main method II (moist chamber incubation) petri dishes with filter paper disks are often used, but it is more convenient to use large rectangular metal or plastic trays with a perforated bottom for avoiding stagnant water. Open trays are all right in high humidity incubators. Otherwise closed transparent polystyrene boxes are necessary. A tray of about $10 \times 25$ cm will accommodate 50 or 100 seeds of most species. The preferable distance between seeds depends on seed size, on the fungi that may be
expected and their spreading capacity, on the prevalence of these infections, and on the
duration and other conditions of the test. When pathogenic infection is rare in a certain
kind of seed, the seeds can be placed close to each other even though the pathogen will
spread from seed to seed during incubation, for in such a case one may count infection
centres instead of infected seeds and regard each centre as caused by one infected seed.
Vacuum counters or perforated boards with holes into which the seeds are shaken
should be used with caution in seed health testing, as they may have a preference for the
light and small seeds and these are often the infected seeds. Accumulation of trace
infections by close spacing can be useful in cases where zero tolerances apply. In the
author's laboratory perspex plates with 50 or 100 holes are used that fit into the blotter
trays, the seeds being fed into the holes from a small cardboard 'table' by means of
forceps. So separate counting of the seeds is avoided and regular spacing is guaranteed.

Blotters with hollows or holes to put the seeds into are useful for keeping round seeds
in place and facilitating water uptake of large seeds. Normally the seeds are not covered
with another blotter. Rolled towel or ragdoll tests are less suited for health investiga­
tion because of the necessities of spacing. Free development of pathogens and in
many cases illumination during incubation.

Plastic dishes are occasionally used for blotter tests. They are preferable to glass, as
polystyrene transmits near-ultraviolet light. But normal petri dishes are shallow and the
lids are soon lifted by the developing seedlings. Also the round dishes cause loss of
incubator space and are not so easily inspected under the stereo-microscope. Covered
plastic trays should be at least 2 cm high. Trays can also be enclosed in polyethylene
bags. It will not be necessary to close the containers when the tests are started with a
sufficient amount of water provided by means of a thick but not water-saturated
blotter layer, and loss of water is restricted by placing them in a high humidity
incubator (about 95% R.H., with only a very weak ventilation). It is best to provide a
sufficient quantity of water by means of a thick blotter or a paper wadding and to place
a thin smooth white blotter directly underneath the seeds which offers a good back­
ground for observation. As adding water during incubation is better avoided and the
blotter surface must not be so wet that development of antagonistic bacteria is strongly
stimulated, it is necessary to use a fairly thick total layer of paper in order to provide
sufficient water for swelling and eventual germination of the seeds, and for develop­
ment of the pathogens notwithstanding some loss of water due to drying, and not at the
same time stimulating the antagonistic saprophytic bacteria on the seeds.

It may be advantageous to stop germination of the seed, for which purpose often
0.1-0.2% 2,4D (2,4-dichloro phenoxyacetate) is used. But this substance is persistent
and consequently dangerous to use in seed testing stations where germination tests
must be made. Killing the swollen or slightly germinated seed by means of deep­
freezing (12 hours at -20°C is normally sufficient) is not dangerous and at the same
time more effective. Some advantages of these procedures are that more seeds can be
placed on the same surface, that they remain neatly in rows and at the blotter level, and
that no sprouts and rootlets will hamper observation at the end of the test. Deep-
freezing is not suited for every seed species and every infection. Moreover both devices have occasionally shown failures.

In most cases identification of species of pathogens, usually fungi, is dependent on their fructification. So incubation conditions have to be such that fungal fructification is promoted. This means high air humidity, either in high humidity incubators or by using polyethylene-enveloped blotter trays or other closed containers. Substrate humidity must be kept somewhat low for restricting development of antifungal saprophytic bacteria, but this can also be realised by wetting the blotters with 250 ppm terramycin or streptomycin solution in place of water. This factor is mainly important for fungal pathogens in small seeds or slimy seeds that quickly imbibe water. For temperate climate fungi temperature should not be above 20°C, as sporulation is often impaired at high temperatures. In certain cases the forecasting value of the blotter test will be increased by using a temperature approximating that of field sowing (Fritz, 1966).

For most infections the factors of incubation temperature and duration and their interaction have been insufficiently studied, so that precision in prescribed methods is often arbitrary. Warm-climate infections may develop better at a temperature above 20°C. For *Drechslera victoriae* Nelson in oat seed 24°C to 28°C has been recommended (Whitehead and Dickson, 1948). Spinach seed is sown in practice in different seasons at different temperature levels. The official germination test is made at 15°C of even 10°C, but in a health test for *Colletotrichum spinaciae* Ell. & Halst. (Rabem. ex Schlecht.) Shoemaker a temperature of 20°C will result in higher infection percentages being found in a shorter time. For *Drechslera graminea* in barley a blotter test temperature of 13–15°C is preferable.

Also the duration of the health test will often differ from that of the official germination test, and certainly the close spacing and early removal of seedlings as practised in germination testing on the Jacobson apparatus is not allowed in seed health testing.

Often a tendency to prefer health testing conditions that will result in the maximum infection percentages being found can be noted, just as in official germination testing the principle of choosing conditions resulting in the maximum germination percentages has been adopted. Nowadays, with the possibilities of eliminating antagonism and reducing host tissue resistance, it may be possible to obtain absurdly high infection percentages that far surpass disease percentages that may develop from the seed in the field (where host tissue resistance as well as soil-borne antagonists in addition to seed-borne antagonists are active). Then a chlorine pretreatment may be applied for decreasing the infection level found and increasing the forecasting value of the test, or a less sensitive method in which natural infection-restricting factors are active can be available. This has to be studied separately for every infection. For quarantine purposes, of course, the most sensitive method is necessary.

Although blotter tests for health are often regarded in the seed testing station as a variation of the blotter test for germination, they should in fact be regarded as an adaptation of the moist chamber method which is often used in plant-pathological work. A blotter or similar substrate is not even a necessity. Seeds may also be incubated
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each in a drop of fluid in hollows in a perspex plate, provided that terramycin is added for keeping the bacterial antagonists in check and deep-freezing is practised for preventing germination (Limmonard, 1968).

Sporulation of many pathogens is strongly stimulated by near-ultraviolet light, preferably in a cycle of 12/12 hours light/darkness (Leach, 1967). Even in cases when a pathogenic fungus does not react in this way, the N.U.V. light may reduce mycelial formation of saprophytes and thus facilitate pathogen observation.

When the seeds have been killed by deep-freezing after a few days of incubation, N.U.V. light is required only during the post-freezing incubation. Then the fungi often develop much more copiously and sporulate more easily.

At inspection a stereoscopic microscope with 25 x magnification will mostly be satisfactory, but for some pathogens up to 50 x is preferable (Pyricularia oryzae Cav.). For blotter/germination tests it may be sufficient only to inspect the apparently diseased seedlings and non-germinated seeds, but for blotter/deep-freezing or 24D tests it is necessary to inspect every seed. Several pathogens may be observed on a single seed, so that the total infection percentage of all pathogens may exceed 100.

The duration of the test must depend on the speed of development of the pathogens that have to be detected. Spread of pathogens notwithstanding seed spacing can be limiting. Early removal of healthy looking seedlings is wrong and early removal of recognisably infected seedlings is risky. Repeated inspection of blotter tests may be necessary, not only when several pathogens with a different speed of development may be present, but also when a single pathogen develops quickly on some seeds and may be masked by other fungi before appearing on other seeds in the same test. Then at the first inspection the infected seeds are marked with an ink pencil. This is very useful in the blotter tests for rice (Oryza sativa L) seed, in which a number of fungal pathogens may be present and quickly developing species may be obscured later on (for example Trichosporon padwickii Ganguly).

It is often possible to dry out blotter tests without impairing identification of pathogens. Most fungal pathogens will maintain their characteristics for quite a time in the dry state. This may be useful in the case of lack of time, or for demonstration purposes.

As a standard blotter test, to be used for infections with which one lacks experience, eight days at 20 C with alternating N.U.V. light is satisfactory. When the pathogen expected is not observed in this way one may try deep-freezing during one night after one or two days of incubation.

In the case of incubation of polyethylene-wrapped trays under horizontal N.U.V. tubes, the lamps will cause the test temperature to rise some 2–3 C above room or incubator temperature. This increase of temperature can seriously reduce sporulation. The same is true for closed petri dishes in illuminated incubators. The lamps underneath the shelves may cause drying out of blotters or agar placed on these shelves. With such a construction good heat insulation is necessary.
II.1. (Lesions etc. after germination in moist chambers)

This heading indicates the observation of seedling symptoms that are sufficiently characteristic for a correct diagnosis. In Doyer’s Handbook (1938) seedling symptoms are heavily relied on, but in recent years it has become increasingly clear that it is often not safe to do so. In cereals the root rot observed in a blotter test after dark incubation is not only caused by *Fusarium* but may also be due to several other fungi, whereas not all pathogenic *Fusaria* cause the symptom. The dark spots on cotyledons of Crucifers may be caused by *Alternaria*, *Phoma lingam* (Tode ex Fr.) Desm. or by threshing injury; also the hypocotyl lesions may be caused by several fungi. In a blotter test with pea seeds *Ascochyta* spp. may cause rather characteristic symptoms, but the agar method is more reliable for these infections. So, for several well known diseases the sub-method is no longer considered satisfactory in many laboratories.

Consequently, although after incubation on moist blotters certain infections already visible on the dry seed may have developed and become more characteristic (*Ascochyta* spp. in peas, *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. & Cav. in beans) and other symptoms may have appeared during incubation (*Fusarium* spp. in cereals, *Septoria nodorum* Berk. in wheat), these symptoms may be more characteristic for the host than for the pathogen. Still, seedling symptoms may be helpful in deciding whether an observed fungus is a pathogen or a closely related saprophyte (*Alternaria hrassicicola* (Schw.) Wilts. or *A. tenuis* Aust. in Crucifers, *Stemphylium radicinum* (Meier, Drechs. & Eddy) Neergaard or *S. botryosum* in Umbellifers, *Pseudomonas lachrymans* (E. F. Smith & Bryan) Carsner in Cucurbits).

II.2. (Pathogen growth after moist chamber incubation)

Identification of pathogens by means of their vegetative growth is seldom safe. For fungi it is preferable to use testing conditions favouring fructification and restricting mycelial formation. Still, the mycelium of members of the ‘roseum’ *Fusaria* may be sufficiently characteristic to recognise this group, though not for distinguishing species. *Rhizoctonia solani* Kühn does not fructify, but its brown hyphae are sufficiently characteristic for identification when the fungus develops from beans, peas and many other kinds of seeds. The yellow exudate of the slime disease of cocksfoot (*Dactylis glomerata* L.) caused by *Corynebacterium rathayi* (E. F. Smith) Dowson, is also sufficiently characteristic.

II.3. (Pathogen fructification after moist chamber incubation)

This sub-method has become increasingly important in recent years, in the first place in consequence of the introduction of fluorescent N.U. tubes in seed health testing (the wave lengths of about 310 nm strongly stimulate fructification of many fungi) and in the second place by the introduction of devices for stopping germination in the blotter medium (such as addition of 2,4-D or deep-freezing), which again emphasise recognition of the infections by means of pathogen development. When cereal or grass seeds infected with *Drechslera* spp. are incubated with N.U. light, the percentages of infection found may be many times higher than those found after incubation in
darkness. When cabbage (*Brassica oleracea* L.) seeds are pre-incubated, frozen, and then again incubated with N.U.V. light, the black leg fungus *Phoma lingam* often forms pycnidia with beautiful pink or violet spore drops. The same method is very satisfactory for *Drechslera* spp. in cereals and grasses and for fungal pathogens of carrots (*Daucus carota* L.). Also, *Phoma valerianellae* Boerema de Jong will not develop its pycnidia easily in a blotter test with growing corn-salad (*Valerianella locusta* (L.) Later.) seedlings, but will do so profusely after the seedlings have been killed by deep-freezing.

Of course, there are pathogens that develop sufficiently characteristically in darkness. One of these is *Helminthosporium sorokinianum* in cereals and grasses. Another is *Colletotrichum spinaciae* in spinach and occasionally in beet, which will at least produce its black setae if not its spores.

II.4. (*Microscopical preparations after moist chamber incubation*)

Making spore preparations for investigation with the high-power microscope is often necessary when learning, but afterwards it can scarcely be accepted in routine testing of commercial seed samples as it is very time-consuming.

Also the microscope cannot be dispensed with when one has to distinguish between different *Ascochyta* spp. in beans, but this will not be required in routine work, or the various *Ascochyta* spp. in peas, which is more important. Often the microscope will be of little avail as the spores formed in the test are insufficiently characteristic. An example is *Didymella lycopersici* Kleb. in *Lycopersicon lycopersicum* (L.) Karsten ex Farwell seed, which has to be distinguished from *Phoma* spp. while itself forming only one-celled spores in the test.

II.5. (*Insect observation after moist chamber incubation*)

After soaking, which often means at the end of an incubation test, it is easy to open the seeds in order to detect internal insects or the damage they have done. This is especially important for bruchid and chalcid infestations. In these pests the pupa, and the adult after emergence from the pupa, remain hidden for some time underneath the seed coat. So whenever the presence of these insects is observed or suspected all apparently sound seeds in the blotter test should be cut open in order to detect insects not visible in a superficial examination. These hidden insects are the reason why inspection of the dry seed for these pests is not sufficient. In a moist medium, however, internally borne insects as a rule will be killed.

The distinction between the groups of insects under II.5. is not a sharp one. In the first place many omnivorous insects cannot feed on sound seeds but follow in the wake of those that can, or they take advantage of some existing injury of the seeds. This is especially true with hulled cereals. Consequently the presence of these ‘secondary’ pests may be considered in connection with a primary injurogen or injury. As, however, the distinction between primary and secondary warehouse pests is rather arbitrary, it is of little consequence in the seed testing laboratory and will normally be disregarded.

Many important warehouse insects can also feed, or even breed, in the field, es-
especially in warm climates. There are also insects that as a rule start development in the field, but continue to attack seed as typical warehouse pests. They may feed in the dry seed as well as in the field crop, and in addition they often are not strongly specialised as to host species. So Acanthoscelides obtectus, the common bean weevil, may act as a destructive warehouse pest in seeds of a number of legumes. Plurivorous insects of this type are more or less intermediate between the omnivorous warehouse pests and those insects, bruchids and chalcids, that feed on a single kind or only a few closely related kinds of seeds. The feeding of the latter, moreover, is restricted in the field to immature seeds although the insects may still be present in the mature dry seeds. Bruchids such as the pea weevil (Bruchus pisorum), the broad bean and the vetch weevils (Bruchus spp.) are examples of this latter group, and also the gall midge Dasineura alopecuri Reuter in foxtail (Alopecurus pratensis L.) seeds, and chalcids in seeds of conifers (Megastigmus spp.), clovers (Bruchophagus funebris), pome fruits (Syntomapsis spp.), etc. Of course all of these insects may have left the seeds before they are tested, so that only the more or less characteristic feeding damage is found.

Also feeding damage may be due to insects which invariably leave the seeds before harvest. The Laspeyresia (pea moth) injury of pea seeds has been mentioned already. Also the gall midge (Contarinia merceri Barnes) in foxtail grass is a good example. In addition we may refer to the Lygus bugs causing injury to many umbelliferous seeds (Flemion and Olson, 1950), beet seeds (Hills and Taylor, 1948; Morrow, 1948), and even to lima beans (Phaseolus lunatus L.) and other species (Baker, Snyder and Holland, 1946), and on the bug injury of cereal seeds (Aufhammer and Hoffmann, 1936/37).

Seed injuries caused by insects which have disappeared may reduce seedling vigour. II.6. (Symptoms of physiological disorders found after moist chamber incubation) The best known example at present is marsh spot of peas, Vicia and Phaseolus beans. Marsh spot is caused by a lack of available manganese in the soil in which the seed crop was grown and thus is physiological in nature. The brown centre of the seed as well as the necrotic aborted plumule, that may or may not accompany the internal browning, are present in the dry seed. However, it is difficult to open the dry seeds and in addition the plumular necrosis is easier to evaluate after some growth. So, although this disease condition had to be mentioned in I.B.6., it is most easily detected by method II.6. (Doyer, 1938: plate XIII).

Often pea seeds after swelling show an internal hollow without necrosis, or a kind of cheesy consistency. This condition has nothing to do with manganese deficiency (Myers, 1948; Perry and Howell, 1965), but is referred to as hollow heart or cavitation. It is a matter of seed vigour. Further we may mention boron deficiency of peas. This is not visible in the dry seed, but causes a decrease in germination that may be corrected by adding borax to the medium (Leggatt, 1948).
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11.7. (Pesticide observation after moist chamber incubation)
A combination of visual examination of the dry seed (I.A.5.), specific chemical tests (I.C.4.) and appearance of the germinating seed will usually prove the presence or absence of a pesticide. Whenever possible sub-methods I.A.5. and I.C.4. must be supplemented by an examination of the germinated seeds and seedlings. If copious bacteria and fungi develop from the seeds and infect the seedlings, it is evident that a fungicide is not present in an effective quantity. Certain treating materials in dust or slurry form colour the seed coats of germinating beans, maize (Zea mays L.) and peas. The formulations of liquid mercurial often contain a special red dye that is clearly visible on germinating cereal seeds or even on the surrounding blotter.

Control of mold growth by fungicides present may be utilised by soaking the blotters in a spore suspension of Aspergillus niger van Tiegh., or a similar fungus that sporulates well on the filter paper. When treated seeds are spaced on the blotter the fungus will fail to develop around the seeds (Schoen, 1965). See also IV.B.

11.8. (Chemical injury after moist chamber incubation)
If typical symptoms of chemical injury appear in the germination test, some pesticide has obviously been applied. The presence of a mercury compound, of lindane or certain other materials can be demonstrated by massing some 300 or 400 seeds closely together on a blotter. The soluble chemical will become concentrated around the seedlings and cause visual symptoms of chemical injury. A well-known type of injury results from the sensitivity of cereals and cucurbits to mercury, evidenced by short stubby rootlets without root hairs and compact swollen sprouts when the seed is germinated on blotters (Lafferty, 1953).

Weed killer treatment of cereals in the field with growth-regulating substances such as 2,4-D may cause injury to the young seed when the treatment has taken place in too late a stage of development. This injury may be revealed in the blotter test by the occurrence of a high percentage of double seedlings (Friesen and Olson, 1953; Olson, Zalik, Breakey and Brown, 1951).

Group III.A includes methods in which seeds are tested by sowing in essentially inert media such as silversand, vermiculite, perlite, molochite, brick grit (Hiltner, 1911). With these media one can make emergence counts and observe seedling symptoms, in this way determining the percentages of healthy, slightly and severely diseased seedlings, and non-germinated seeds. This can be regarded as an intermediate between laboratory tests and sowings in field soil, but can scarcely be regarded as a satisfactory method of health testing. In such media pathogen observation and identification is seldom possible because free observation of the seeds and seedlings is prevented. Even when they are dug out at the end of the test and washed to remove adhering particles of the medium, they still are not so easy to observe as are the clean seedlings from a blotter test.

Moreover, because with germination under a covering layer of the mediums conditions are not conducive for fungus sporulation. It will rarely be possible to observe and identify fungus fructifications. Of course, diseased host parts from these tests may
be surface-disinfected and incubated on moist blotters under conditions favourable for
the appearance of the pathogen, or plated on nutrition agar, and in this way additional
information can be obtained.

Phytotoxic injury in consequence of pesticide treatment is best evaluated in a soil
type medium. In such a medium, owing to the dilution and adsorption of the pesticide,
as in the field, the injury is normally less than in a blotter or even a sand test (Thomson,
1954).

III. B. (Examination of seedlings after emergence in non-sterile soil)
Examination of seedlings after emergence in non-sterile soil is not attractive for routine
seed health testing but there may be no good alternatives. Non-sterile soil is in-
sufficiently standardised, so that the results obtained with it are often unfit for com-
mercial and international use. Temperature-conditioned soil tests may be used instead
of field sowings for comparison with laboratory test results. They are not subject to
many hazards that may affect field trials and permit a more detailed observation. It is
best to adapt soil test temperature to the normal average sowing temperature of the
crop studied. When light conditions are satisfactory soil tests can be kept on for a
considerable time; emergence counts can be made and disease symptoms studied. Seed-
borne infection causing pre-emergence damping-off is always difficult to study in soil
tests. Still, soil tests can be useful for comparison of the infection levels obtained with
different laboratory methods. When seedling disease symptoms are chiefly post-
emergence, soil tests can be used for determining correlation coefficients between
laboratory test results and soil test observations, with the purpose of comparing the
practical value of various laboratory test methods.

IV. A. (Incubation on agar media)
Low pH media such as potato dextrose agar (PDA) or malt extract agar (MA) are best
suited for 'field fungi', to which category belong the pathogenic fungi normally studied
in seed testing as well as the common saprophytes. The same media with 7 1/2% or 10 %
NaCl or a high concentration of sugar added are excellent for the xerophytic 'storage
fungi', e.g. Aspergillus spp., that are mainly responsible for deterioration of seeds under
unfavourable conditions of storage (Christensen, 1957). Neutral or alkaline media,
such as Bactonutrient agar or Bacto-peptone agar, are better suited for bacteria. By a
suitable choice of medium certain groups of micro-organisms can thus be emphasised
and antagonism restricted.

Whether surface disinfection of the seed will be necessary is dependent on the seed
species and the conditions under which it has matured. For most kinds of seeds with the
agars mentioned a hypochlorite pretreatment will be necessary. Hypochlorite solutions
for household use may be suitable, diluted to 1% or 0.5% NaOCl. The solution is
poured off after for instance 10 minutes and the seeds are usually plated after draining
but without washing, using flamed forceps.

One may use ready-made agars (Difco, Oxoid) or prepare PDA from instant
mashed potatoes (Lacy and Bridmon, 1962), an agar with V8 tinned fruit juice, etc. The
dissolved nutrition agar is sterilised and poured into sterile petri dishes. Plastic petri dishes are normally bought sterile in plastic bags and thrown away after use. Glass dishes are far more expensive, and they have to be cleaned and sterilised with dry heat for repeated use. Normal glass is non-transparent to U.V. light. For steam sterilisation of agars half an atmosphere overpressure during half an hour is normally sufficient.

When testing for fungi it may be advisable to add a trace of terramycin (about 50 ppm) or streptomycin (some 100 ppm) to the nutrition agar after sterilisation for controlling bacterial growth.

It has some advantages to practise gas sterilisation of poured agar dishes (Watson, Curley and Huber, 1966). Then the nutrition agar is only heated to dissolve the ingredients, and the terramycin or streptomycin added. The dishes are poured and when the agar is solid a pile of dishes is sealed into a polyethylene bag together with a little corked bottle containing fluid propylene oxide. For 20 dishes with nutrition agar containing streptomycin 10 ml of propylene oxide is sufficient. After sealing, the cork is removed from the little bottle through the plastic bag, the fluid evaporates and sterilises the agar dishes after which the gas disappears spontaneously through the polyethylene. After two to three weeks, depending on temperature, the gas has gone and the dishes are ready for use. The procedure allows of large-scale work under non-sterile conditions. It is possible always to have a store of poured dishes ready for use, protected against re-contamination and drying-out in their polyethylene bags (Limonard, 1967). Care should be taken as propylene oxide is inflammable and explosive, but it is far easier to handle than the twice as effective but also more volatile ethylene oxide.

For plating the seeds one needs a clean transfer room. Ideal is a room with an electronic air cleaning device (Limonard, 1967), which enables hundreds of agar dishes to be handled per day without appreciable loss through airborne fungi (often originating from other tests).

The number of seeds per dish depends on seed size, the pathogens to be expected and the infection level. Normally 5, 7 or 10 seeds are used per 9 cm dish.

For incubating temperate climate seeds with fungal infections a 20°C incubator is satisfactory. Fructification of fungi can be stimulated by applying fluorescent U.V. light. This can be restricted to the last few days of incubation, for instance with five or six days in darkness followed by two or three days at 20°C with U.V. light in a cycle of 12 hours light and 12 hours darkness.

For determining pathogen percentages naked-eye inspection of obverse and reverse of the fungus colonies is rarely satisfactory. One has to use the stereoscopic microscope with through-falling light and about 20-25 × magnification, scrutinising the colonies and also the seeds themselves for sporulating fungi. Often mixed colonies occur, or antagonists prevent a pathogen from growing on the agar so that it only succeeds in forming a few sporophores on the seed coat. Thus nowadays the preparation of the agar test is much simplified but their inspection is more difficult and time-consuming than was formerly thought satisfactory.

For *Septoria nodorum* and *Fusarium* spp. in wheat seed the use of 0.2% oxgall or
similar growth retardants in the PDA is useful. Then the colonies form little mycelium and sporulate profusely, so that they are easily identified (Miller, Peers and Neal, 1951). 10 seeds can be easily plated in a 9 cm dish, and some delay in inspection due to lack of time on the official inspection day does not matter.

A recent development is the use of specific agars in seed health testing, such as Nash and Snyder's peptone-PCNB agar for Fusaria (1962). This is so specific for species of the fungus genus *Fusarium* (and *Cephalosporium*) that sterilisation is not even necessary provided one starts with reasonably clean petri dishes. Sporulation of Fusaria on this nutrition is abnormal, but one soon learns to distinguish between *F. nivale* (Fr.) Ces. and the *roseum* group with the help of transfers to PDA that sporulate normally.

When time for inspecting the dishes is lacking one can sometimes let them dry out and store them until time is available.

**IV.A.1. (Plating after special pretreatments)**
The most common pretreatment is one with 1% NaOCl for 10 minutes, after which the seeds are plated directly. Some kinds of seeds are well cleaned by such a pretreatment, so that inspection of the dishes is easy and magnification is not needed. An example is the *Ascochyta* spp. in pea seeds.

In Canada the agar method with pretreatment was used on a large scale during many years for a wide range of seeds. A thorough pretreatment was given using 2% chlorine up to 20 minutes.

As indicated earlier it is normal to plate the chlorine-pretreated seeds without washing them. If, however, the agar contains an antibiotic it may be preferable to rinse the seeds in water, so that the chlorine will not oxidise the trace of antibiotic.

As a standard method a chlorine pretreatment of 10 minutes in 1% NaOCl, followed by incubation during eight days at 20°C, with alternating N.U.V. light during the last two or three days, may be adopted. This will be satisfactory for most infections for which the agar method is suited.

When specific agars are used a chlorine pretreatment is not decidedly necessary, but may be applied for bringing the infection percentages down to a reasonable level by eliminating the very slight infections of individual seeds.

The dry heat treatment, one hour at 100°C, developed by Malone, (1962), has only a limited applicability. It should not be used for seeds with a high moisture content.

**IV.A.2. (Plating without surface disinfecion)**
This was first done on a large scale for flax seed by the Belfast group using malt extract agar (Muskett and Malone, 1941). In flax a variety of seed-borne fungal pathogens may occur but otherwise the smooth seeds are very clean. Consequently a chlorine pretreatment is normally superfluous. The colonies are sufficiently characteristic and are little hampered by saprophytic fungi, so that the unaided eye is usually sufficient for identification. But for many other kinds of seed superficial disinfection is necessary.
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IV.B. (Examination for the presence of fungicides on the seeds)

When the purpose is to test seed samples for the presence of chemicals, the seeds are spaced on agar sheets inoculated with a fast-growing species of bacterium or mould. After a few days of incubation the clear mould-free zones surrounding the treated seeds are measured. By comparison with correctly treated control samples it is possible to verify statements concerning treatment of the seed lot with a certain dosage of a certain substance. Uniformity of distribution of the fungicide can also be tested in this way.

The agar sheets are prepared on a glass surface. An even distribution of the fungus spores or bacterial cells in the agar may be difficult to obtain, especially if one wishes to avoid distributing spores all over the laboratory. Originally a Penicillium sp. was used for the purpose, the spores being blown over the agar (Machacek, 1950). Later workers have turned to Glomerella cingulata (Stonem.) Spauld & Schrenk or to the hay bacillus. With the latter an even distribution in the agar is easy to obtain because of its heat resistance, so that it can be added to the still quite hot agar before pouring it on to the glass (Arny, 1952; Moore and Olin, 1952). The Scottish workers originally used Stemphylium tilisci Tengwall but more recently a Phoma sp.

It is difficult to pour a large fungus-agar plate of uniform thickness with the spores evenly distributed. Rectangular glass plates with glass strips glued along the borders can be used. It is advisable to warm the plates before pouring the agar. Also the water used for making the spore suspension should not be cold. The glass plate should be placed precisely horizontal, which can first be tried with water. It can be made sterile with 50% alcohol.

The method has some quantitative value and is excellent for checking whether seed has been fungicide-treated, whether the coverage is uniform, and by comparing with checks treated in the laboratory with the same fungicide, to verify whether the dosage has been right. Conclusions can be drawn concerning the efficiency of machinery for seed treatment. In addition the method is valuable for research purposes. For evaluating the effectiveness of a certain pesticide treatment against a certain disease the method is, however, unsuited. Here blotter or agar tests (in the case of seed-transmitted pests), or soil tests (also fit for soil-borne pests) must be recommended.

Methods in group V include the growing of plants beyond the seedling stage and examining them for disease symptoms. It can be the most convenient, indeed often the only possible, method for determining whether fungi, bacteria or viruses are present in the seed. The plants may be grown in the greenhouse or in the field, possibly in connection with variety trials (as in Sweden) but protected against chance introduction of pathogens. Seeds from the suspected sample may themselves be sown (V.A.), or inoculum obtained from the seeds may be used for inoculating healthy host plants (V.B.).

Method V.A. is essentially based upon the same principle as applied to seedling investigation. But the plants are grown until a later stage, sometimes even until maturity, and then inspected for signs and symptoms of disease.

This is a branch of phytopathology in which the current handbooks and extension
publications are very helpful. Of course precautions have to be taken to prevent infection of the plants from other sources than the seed. Good examples of the application of this method are the smut fungi and certain virus diseases (common bean mosaic, lettuce mosaic). For insect-transmitted viruses special glasshouses, or cages for individual seed samples, are required to prevent infection of the plants by virus-transmitting insects. Disadvantages are that the method is time-consuming, expensive and often uncertain. It is widely used in the Scandinavian countries, where field inspection and seed sample testing and field trials alternate, and fields are refused certification if the test plots show too much of certain seed-borne diseases. Thus in these countries the testing for health condition is partly performed in the field instead of in the laboratory and the disadvantages mentioned are minimised because the inspection for certain diseases is part of the field inspection which is required in any case.

Of course in test plots control of environmental conditions is largely impossible. This is a serious drawback, for the appearance of infections can be dependent upon conditions such as temperature, soil moisture and seed vigour. For other diseases chance infection from surrounding sources is an important risk and the spreading of these diseases is often much influenced by external conditions. These disadvantages, however, are partly overcome in a ‘closed system’ of alternating field and seed inspection by shifting the health evaluation of the seed from the following to the preceding crop: if the test plot or the field is seriously infected, then certification of the harvested seed is refused. However, parent crop inspection neglects the possibility of disease escape of the seed. The reliable use of method V.A. is in practice restricted to only a limited number of diseases. So it will often be necessary to supplement it with health investigation of the seed in the laboratory.

Rohloff (1967) used a growing-on test for demonstrating mosaic virus in lettuce (Lactuca sativa L.) seed samples. The method is simple but time-consuming, space-taking and expensive.

In method V.B. suspicious micro-organisms are isolated from seeds and seedlings that have been found to be diseased, and identified by the symptoms they cause in inoculation experiments using healthy seeds or plants. This method is growing in importance now that seed pathologists are starting on the more difficult infections (bacteria, viruses), for instance bacterial bean diseases. Warne (1944) used it to prove that Plasmopara brassicae Woron. may be seed-borne in swedes (Brassica napus L.) and Kerling (1952) to demonstrate that Fusarium oxysporum Schlecht. ex Fr. f. pisi race 1 is transmitted in pea seed. But their methods are too time-consuming for routine testing of series of commercial samples.

4. General evaluation of testing methods, mainly for fungi

Some practical remarks in connection with health testing of seeds may be added here. In the foregoing section the methods, their scope and restrictions, advantages and disadvantages have been discussed. This fourth section may serve to simplify the procedure for seed analysts, who wish to make a start with this work but feel confused.
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by the many diseases known to be seed-borne (Noble and Richardson, 1968) and the number of methods available.

If a routine seed analyst starts with health investigation he will naturally favour methods similar to those to which he is accustomed. These are the possibilities connected with the investigation for purity and germination, e.g. certain methods of groups I. and II. Of course, it will be necessary to acquire some plant-pathological knowledge but then it will soon be possible to recognise sclerotia etc in the inert matter as probably belonging to certain pathogens (I.A.1.), to suspect infection when certain types of spots occur on the seeds (I.A.2.), and to recognise certain insects or symptoms of insect damage (I.A.4.). In addition the possibility will be discovered of detecting fungal fructifications (I.A.3.) with a hand lens.

Some of these possibilities are very satisfactory, in particular I.A.1. Others are only of some value as a preliminary indication of the pathogenic conditions of the seeds, e.g. I.A.2., I.A.3., I.A.4. To the diseases and pests of this second group an incubation test as listed under II and IV is better adapted, and a seed analyst accustomed to germination tests will be inclined to turn to a special blotter test for health (II). But both the blotter and the agar methods for health testing require a plant-pathological education. They are too complicated and judgment is too difficult for workers without such knowledge to use them successfully. Also these methods require special equipment. In short, health testing has to be done by specialised analysts in a separate department.

Provided the knowledge and equipment is available, both the blotter and the agar method will prove their value in seed testing. Different infections require different methods, and although it may be difficult to indicate which method is the best for a certain infection it is not so difficult to exclude certain methods as unsatisfactory. There are cases in which it can be indicated which method is better, but for most infections even the information required for a justified choice between the two main incubation principles is still lacking.

5. Special methods for bacteria

The methods in groups I to V have a limited value for bacterial infections of seeds. In the case of bacteria it will often be necessary to replace them, or to confirm their results, by some highly specialised methods.

In most methods for bacteria it may be necessary to apply a light disinfectant pretreatment for eliminating saprophytic bacteria, but this may also reduce pathogenic bacteria situated on or near the seed surface (*Xanthomonas malvacearum* (E. F. Smith) Dowson in cotton (*Gossypium* spp.). Also it can be useful to accumulate the pathogen by a soak pretreatment using sterile water (Schaad, 1976, for *X. campesiris* (Pammel) Dowson in cabbage), nutrient broth (Otta, 1977, for *Pseudomonas syringae* van Hall in...
wheat), or broth with selective compounds added (Guthrie, 1970; crystal violet for bean bacteria). The seeds may be used whole or ground. The information obtained is usually only quantitative, but this may be satisfactory in the case of quickly spreading bacterial diseases.

Dry seed inspection has some value, e.g. in the case of bacterial bean pathogens. N.U.V. light may be helpful (Wallen and Sutton, 1965; Wharton, 1967).

A simple blotter or sand test is sometimes useful for bacterial infections, for instance Corynebacterium rathayi in cocksfoot (Skou, 1965), P. lachrymans in Cucurbita (Volcanci, 1966) and X. malvacearum in cotton (Halfon-Metri and Volcanci, 1977). Srinasvan, Neergard and Mathur (1973) pretreated cabbage seeds with aureofungin for eliminating fungi as causes of seed rot, and then had the seeds germinating on water agar for observing rot caused by X. campestris.

On common bacterial agar-plates colony appearance may be indicative, for instance the brown colour in the case of X. phaseoli (E.C. F. Smith) Dowson var. fascians in beans (Wallen and Sutton, 1965). Xanthomonas spp. dissolve starch in agars containing soluble starch (Schaad and Kendrick, 1974, with X. campestris). Also selective agars are used (for instance Suprianan and Tantera, 1972 with X. oryzae (Uyeda & Ishiyama) Dowson in rice). Fluorescence on King’s Medium B was used by Wharton (1967) for P. phaseolicola and by Otta (1977) for P. syringae in wheat.

A growing-on test with suspected seed may require a climate room for providing suitable standardised conditions. Even so an infection may be present without causing symptoms.

Host plants may be used as test plants for seed extracts or for checking provisional results obtained in crude methods. Already in 1935 Wilson used green bean pods for P. phaseolicola. Saettler (1971) tested for various bean bacteria on young bean plants. Thr (1969) worked with tomato plantlets in a test for Corynebacterium michiganense (E. F. Smith) Jensen. However, the quantity of pathogenic and saprophytic bacteria in the material to be used for inoculation may be crucial for the result (Klement, 1970).

The special methods for bacteria can be used either directly or for confirming observations made in crude methods. Foremost among the special methods are those based on serology. For these, training at and co-operation with a plant-pathological laboratory nearby is necessary. Ready-made serum may be obtainable from a central laboratory.

In method VI.A.1., the double diffusion technique, the bacteria are killed to obtain soluble antigen and placed in wells in a plate of purified agar. A central well in a circle of such wells is provided with a specific antiserum. After incubation a precipitation line between the central well and one of the surrounding wells will indicate the presence of the pathogen in the latter. The serum must cover all the strains of the pathogen that may occur. The concentration of the bacteria has to be fairly high for a distinct reaction. The actual test is quick and takes little space. Guthrie (1970) used it to test beans for P. phaseolicola.

In VI.A.2., the slide agglutination method, a droplet of bacterial suspension is mixed on a glass slide with a droplet of antiserum. Presence of the specific bacterium will result
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in a clouding that is visible under low magnification. The antiserum must be very specific to avoid cross reactions. The method is more sensitive than L.A. It was used by Lovrekovich and Klement (1963) and Taylor (1970) for bean bacteria.

For VI.A.3., the immunofluorescence technique, the antiserum or the anti-rabbit antiserum is labelled with a fluorescent compound and then added to a bacterial preparation on a glass slide. With a fluorescence microscope even a single bacterial cell can be detected. The method requires a very selective antiserum in order to discriminate between specific and non-specific reactions. It is quite sensitive and can be used with crude bacterial preparations. Trigalet and Rat (1976) applied the technique for detecting *C. michiganense* and *P. phaseolicola*. Also Coleno (1968) and Köhn (1970) used it for the latter.

Quite another approach is the use of bacteriophages in the so-called phage-plaque technique VI.B. Bacteriophages cause lysis of their host bacteria but they are not always suitable as their host range may be either too narrow, only including certain strains of the pathogens, or too broad, also covering related species. Often a mixture of phage strains will be required. When a phage suspension is mixed with a suspension containing its host bacterium the phage particles will multiply. Then the bacteria can be centrifuged off and the supernatant mixed with nutrient agar containing the reference bacterium. Then plates are poured and incubated. A substantial increase in the number of phage particles and consequently in the number of lysed spots ('plaques') due to incubation of the mixture of phage and unknown bacteria indicates the presence of the pathogen. Katznelson and Sutton (1951), Katznelson, Sutton and Bayley (1954), Wallen and Sutton (1965) and Ednie and Needham (1973) used this method for *X. phaseoli* and *X. phaseoli* var. *fassans*. For certain strains of *P. phaseolicola*, Taylor (1970) found his phages to be too selective. The method is very sensitive and does not require pure cultures of the bacterium to be prepared from the seed. Ednie and Needham (1973) detected one infected bean seed in 6400.

These qualitative methods can be more or less quantified by trying them on decreasing numbers of seeds (sequential analysis). Taylor (1970) used this approach for *P. phaseolicola*.

6. Special methods for viruses

For viruses also the general methods have little or no diagnostic value. The dry seeds when virus-infected may be shrunken, discoloured or lighter in weight. Pea seeds suspected of carrying early browning virus can be selected (Bos and Van der Want, 1962). Mosaic-infected lettuce seeds can be accumulated in the light blowing fraction (Ryder and Johnson, 1974). In these cases a more specific test has to follow.

In a blotter test the stripe mosaic infection of barley can be determined (Phatak, 1974) but a growing-on test in soil is often preferred (Hampton, Sill and Hansing, 1957). Optimal plant growth conditions usually favour symptom expression and should be standardised. Often a temperature of 22 to 25 °C is recommended. Also light conditions can be important (Hampton *et al.*, 1957, for barley stripe mosaic; Rohloff, 1964.
for lettuce mosaic). The environment used should be aphid-free (Bos, 1967). The controlled environment method for lettuce mosaic is an example of growing plantlets for virus symptoms from the seeds (Rohloff, 1965).

Masking of virus symptoms in a growing-on test is not uncommon. For fast-spreading viruses inspecting large numbers of seeds is required for detecting trace infections. This may make the use of test plants preferable. Soaked or pre-germinated seeds are ground in buffer solution containing additives that prevent virus inactivation. The juice is used for inoculating carborundum-dusted test plant leaves. Quantz (1957) tested bean seeds for common mosaic on detached leaves of *Phaseolus vulgaris* 'Top crop'. Incubation conditions are very important for symptom expression and not all strains of the virus cause the symptoms (Drijfhout, personal communication). Bos and Van der Want (1962) tested selected suspect pea seeds for early browning virus on detached cucumber leaves. Marrou and Messiaen (1967) and Kimble, Grogan, Greamethead, Paulus and House (1975) used *Chenopodium quinoa* Willd. plants for indexing lettuce seed for mosaic, and Phatak (1974) found even symptomless plantlets to react positively on this test plant. Blaszczak and Kowalska (1966) tested yellow lupin (*Lupinus luteus* L.) for bean yellow mosaic on *Chenopodium amaranticolor* Coste & Reyn. Walkey and Whittingham-Jones (1970) used the same test plant for strawberry latent ringspot virus in celery (*Apium graveolens* L.) seedlings. Although test plant and growing-on methods give much information, confirmation of virus identity may require other methods.

The special methods for viruses are closely related to those for bacteria. Much is still in the research stage. VII.A. electron microscopy, was used by Phatak (1974) for detecting bean common mosaic and barley stripe mosaic in plumules of infected seeds. B. histochemical tests, have been used for demonstrating viral inclusions in epidermal leaf tissue by staining with 1% phloxine in water (Phatak, 1974). Also staining with a fluorochrome is possible. Phatak and Summanwar (1967) suggest the direct vital staining of the seed, which possibility might obtain practical value in seed testing.

VII.C. serology, may be useful when differentiating a virus from related viruses on indicator plants is not possible.

The double diffusion technique (VII.C.1) is described under Methods for Bacteria. As antigen the whole virus particles are used. Extracts of seeds, seedlings or leaves of grown plants may be tested. The method is well suited for isometric viruses as these diffuse quickly into agar gels whereas filamentous viruses are too slow and have a tendency to aggregate. Phatak, Mathur and Neergaard (1971) used double diffusion for detecting tobacco mosaic in tomato seed. For isometric viruses such as cucumber mosaic, tomato blackring etc seed testing has not yet been introduced. Langenberg and Ball (1972) used chemical degrading of filamentous viruses preceding the double diffusion test. Slack and Shepherd (1975) applied a radial single diffusion technique in testing for barley stripe mosaic. They placed leaf parts of seedlings on agar plates containing antiserum and a detergent for dissociating the virus particles. Other refinements such as autoradiography of labeled antigens (Schlegel and Hudson, 1969) may
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be applicable in future. It has the great advantage of the possibility of checking many samples at a time.

Agglutination methods (VII.C.2.) can be unreliable because of non-specific reactions, even when the antibodies have been attached to inert carrier particles such as bentonite. A recent improvement is the introduction of uniform latex particles for carrying antibodies. So soybean mosaic was easily detected in extracts of seedling plumule and even in non-germinated seeds (Phatak, 1974). Barley stripe mosaic was studied by Lundsgaard (1976) in this way.

Immunofluorescence (VII.C.3.) is also used for virus detection. The host tissue suspected of containing the virus is flooded with antiserum, and the virus if present binds part of the antibodies. Then the infection is seen as a fluorescent area under the fluorescence microscope. When an anti-antiserum (prepared for instance, via a rabbit and then a sheep) is conjugated with the fluorescing compound the reaction may be more distinct because more gamma-globuline molecules will be attached to each antiserum molecule. Murayama and Yokoyama (1966) applied this technique to plant viruses.

Testing of squash preparations of seeds or seedlings is possible (Phatak, 1974).

In the enzyme-linked immuno-sorbent assay (E.L.I.S.A.; VII.C.4.) the antiserum is adsorbed to a polystyrene microtiter plate, then the virus is added and attached to the antiserum molecules. After washing, enzyme-labelled antiserum is added and bound to the virus. After washing again for removing surplus antiserum the enzyme substrate is added. The enzymatic reaction rate is a measure for virus concentration. It can be easily followed with a simple colorimeter. Recently this technique has been used for plant viruses (Clark and Adams, 1976; Clark, Adams, Thresh and Casper, 1976; Bossennec and Maury, 1977). The method might be promising for seeds and seedlings.

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