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Forest tree seed
health
for germplasm conservation

J.R. Sutherland, M. Diekmann and P. Berjak, editors

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Introduction to the Series

The Technical Bulletin series is targeted at scientists and technicians managing genetic resources collections. Each title will aim to provide guidance on choices while implementing conservation techniques and procedures and in the experimentation required to adapt these to local operating conditions and target species. Techniques are discussed and, where relevant, options presented and suggestions made for experiments. The Technical Bulletins are authored by scientists working in the genetic resources area. IPGRI welcomes suggestions of topics for future volumes. In addition, IPGRI would encourage, and is prepared to support, the exchange of research findings obtained at the various genebanks and laboratories.

The International Plant Genetic Resources Institute (IPGRI) is an autonomous international scientific organization, supported by the Consultative Group on International Agricultural Research (CGIAR). IPGRI's mandate is to advance the conservation and use of genetic diversity for the well-being of present and future generations. IPGRI's headquarters is based in Maccarese, near Rome, Italy, with offices in another 19 countries worldwide. The Institute operates through three programmes: (1) the Plant Genetic Resources Programme, (2) the CGIAR Genetic Resources Support Programme and (3) the International Network for the Improvement of Banana and Plantain (INIBAP).

The international status of IPGRI is conferred under an Establishment Agreement which, by January 2001, had been signed and ratified by the Governments of Algeria, Australia, Belgium, Benin, Bolivia, Brazil, Burkina Faso, Cameroon, Chile, China, Congo, Costa Rica, Côte d'Ivoire, Cyprus, Czech Republic, Denmark, Ecuador, Egypt, Greece, Guinea, Hungary, India, Indonesia, Iran, Israel, Italy, Jordan, Kenya, Malaysia, Mauritania, Morocco, Norway, Pakistan, Panama, Peru, Poland, Portugal, Romania, Russia, Senegal, Slovakia, Sudan, Switzerland, Syria, Tunisia, Turkey, Uganda and Ukraine.

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Cover: Pod of *Pterocarpus macrocarpus* (Vietnam). Jarkko Koskela, IPGRI.

Contents

Preface

The conservation and use of forest genetic resources worldwide poses several challenges to scientists, policy-makers and, in particular, to local stakeholders interested in long-term strategies to manage these biological resources in a sustainable manner. The vast diversity of tree species, many of which are still unknown, the high level of threats and the increased demand for forest products require prioritization of actions, clear indications for research and development, and strategies to mitigate the current trends in the depletion of forest resources.

The strategy of conservation 'through-use' of forest genetic resources is a very important alternative to an *in situ* approach and, as such, is to be promoted and developed. However, basic knowledge and understanding of species' reproductive biology, seed production, seed quality and health aspects, limit the use of a larger number of species in important activities such as restoration, rehabilitation, agroforestry and on-farm conservation practices. Increasingly, the use of forest genetic diversity in research and breeding requires a greater movement of germplasm.

This Technical Bulletin, prepared by Drs J. R. Sutherland, M. Diekmann and P. Berjak, all well-known scientists in their respective areas of specialization, aims to breach some of the knowledge gaps in forest seed biology and technology and, more importantly, to contribute to future research on priority forest seed health aspects. This is an area of extreme importance for an effective and safe use of existing diversity of tree species in either agroforestry projects or in conservation of genetic resources in managed landscapes programmes, and also to widen the scope of this diversity in the activities listed above. Protocols for fungi and virus detection using different techniques are also presented and discussed extensively.

In addition, this Technical Bulletin aims to increase awareness amongst technical staff involved in conservation and use activities. To this end it presents state-of-the-art tools for the identification of the most important tree seed pathogens and provides clear and ready-to-use molecular-based tools for the screening of fungi and virus in seeds.

We hope that with this publication some of the existing gaps in the knowledge of forest seed health management are addressed in such a way that seed scientists, pathologists and foresters may fully benefit from existing forest biodiversity, and contribute to their effective conservation and efficient and safe use.

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1 Introduction

Everyone who works with orthodox and/or recalcitrant tree seeds should be concerned with seed health issues. Seed health analysts would like to know if germination failure is the result of seed-borne pathogens, whereas foresters and seed dealers often discuss the importance of moulds on the quality of the tree seeds they collect or sell. Reduced germination is of particular importance for genebanks storing forest seeds. Forest nursery managers are interested in seed-borne pathogens affecting seed germination or causing disease in their crop. In tree improvement programmes, pathogens may be transported long distances along with the plant germplasm used. Plant quarantine officials should know if seeds being moved domestically or internationally harbour pathogens of importance to local forests.

A number of publications list micro-organisms of tree seeds (e.g. Ivory and Tompsett 1994; Mittal *et al.* 1990; Mohanan and Sharma 1991; Prochazkova and Jancarek 1991; Sutherland *et al.* 1987), but there is not a publication dealing solely with forest tree seed health testing. Thus, the purpose of this publication is to assemble several protocols for detecting pathogens of conifer and hardwood seeds.

Most of the reports on seed-borne pathogens of forest trees, deal with fungi. However, data on seed-borne viruses of trees are accumulating and, consequently, we have included a section on viruses of hardwood seeds. Although numerous species of bacteria may be detected on tree seeds (Mittal *et al.* 1990), fruits and cones, little is known about their role in seed health. Some, like the bacterial wilt pathogen, *Ralstonia* (*Burkholderia*/ *Pseudomonas*) *solanacearum*, of *Eucalyptus* spp. (Ciesla *et al.* 1996), may eventually prove to be seed-transmitted, whereas other seed-borne bacteria might be beneficial, i.e. in stimulating seedling growth, as occurs with many soil-borne bacteria (Chanway 1997).

Considering the limited knowledge of the role of bacteria on tree seeds, the general protocols described here, complement those procedures recommended for individual pathogens on specific seed species, published by the International Seed Testing Association (ISTA). Also included in this technical bulletin, are assays for several pathogens and species of tree seeds, which have not been previously covered. We have also included a section on non-orthodox or recalcitrant tree seeds, contributed by an expert in the field, Dr Patricia Berjak, University of Natal, South Africa, to cover this important group of forest tree species. She discusses different disease

detection methods and other procedures for pathogen detection and control.

We expect that this information will be useful to seed analysts and general diagnosticians working in genebanks, plant quarantine, and other research facilities, especially in countries where pertinent references may not be available. No attempt has been made to list all of the pathogens found on tree seeds. Refer to Mittal *et al.* (1990) and Richardson (1990) for such information.

Whenever possible, taxonomic references are given for identifying fungi. We also recommend consulting 'A Literature Guide for the Identification of Plant Pathogenic Fungi' (Rossman *et al.* 1987) which gives taxonomic references for identifying plant pathogenic fungi. Many seed-borne fungi produce only an anamorph (asexual) stage. Barnett and Hunter (1998) give an excellent taxonomic key to the genera of these fungi. General laboratory procedures or recipes for commonly used culture media such as PDA are not covered in this publication. Details can be found for example in Johnston and Booth (1983) or Hawksworth *et al.* (1995). Although we describe several techniques for isolating specific fungi or groups of fungi, particularly the use of selective media, many seed-borne fungi are easily isolated from surface-sterilized seeds plated onto water agar, potato–dextrose agar (PDA), malt agar (Uniyal and Uniyal 1996), or other standard culture media (Diekmann and Sutherland 1998). Try these less-complicated procedures before moving on to more specific protocols.

2 Fungi and seed storage

2.1 Orthodox seeds

Most temperate tree species produce seeds with an orthodox seed storage behaviour. Orthodox seed storage behaviour is defined by Hong *et al.* (1996) as "mature whole seeds (which) not only survive considerable desiccation (to at least 5% moisture content) but their longevity in air-dry storage increases in a predictable way by reduction in seed storage moisture content and temperature". Recalcitrant seeds in contrast, are "unable to tolerate more than a limited amount of desiccation, for example to moisture contents in equilibrium at 20°C, with about 96–98% relative humidity". In between these two categories, are some species with an intermediate storage behaviour, which "are able to tolerate desiccation to seed moisture contents in equilibrium at 20°C with about 40–50% relative humidity but where further desiccation often reduces viability and always results in more rapid deterioration in subsequent hermetic storage the more the seeds are dried below this value". Examples for trees with orthodox storage behaviour are *Fagus, Fraxinus, Pinus* and *Prunus*. Recalcitrant species are cacao (*Theobroma cacao*), rubber (*Hevea brasiliensis*), durian (*Durio zibethinus*) and jackfruit (*Artocarpus heterophyllus*); temperate recalcitrant species are oak (*Quercus robur*), maple (*Acer saccharinum*) and horsechestnut (*Aesculus hippocastanum*). Coffee (*Coffea* spp.), African oil palm (*Elaeis guineensis*), papaya (*Carica papaya*) and several *Citrus* species belong to the intermediate group.

2.2 Non-orthodox seeds

Seeds that may be collectively categorized as non-orthodox, especially those commonly described as being recalcitrant, differ from orthodox types in terms of the final stages of pre-shedding maturation and, notably, in their post-harvest responses. Most orthodox seeds undergo maturation drying as the final phase of their pre-shedding development and will come to water content equilibrium with the relative humidity (RH) of the atmosphere. Even where this is not the case, after harvest orthodox seeds will tolerate a substantial degree of further dehydration (to ca. 5% moisture content [dry mass basis]), and are storable for predictable periods under defined conditions of RH and temperature (Ellis and Roberts 1980). Seeds of any species that do not behave in this way are considered to be non-orthodox (Berjak *et al.* 1989). While most of the species considered elsewhere in this publication are strictly orthodox, this section deals with those that are not.

The original, formal definition of seeds as being orthodox or recalcitrant and, indeed, the introduction of these terms was based on their storage behaviour (Roberts 1973), which is a manifestation of post-harvest seed physiology. When they are shed, not only are recalcitrant seeds characterized by relatively to very high water contents, but they are also actively metabolic (Berjak *et al.* 1989). In this condition, such seeds will withstand only very restricted dehydration before severely damaging or lethal effects occur, and consequently are described as being desiccation-sensitive (e.g. Chin and Roberts 1980). This is, in fact, the major criterion by which seeds of particular species are categorized as being recalcitrant. Although there is an enormous gap in dehydration response between recalcitrant and orthodox seeds, thus far only one further category has been formally defined—that comprised by seeds which will withstand a substantial degree of dehydration, but not to as low water contents as will orthodox types: these have been described as showing intermediate storage behaviour (e.g. Ellis *et al.* 1980), and those of tropical origin may, especially in the dehydrated condition, be adversely affected by chilling (Hong and Ellis 1996).

Despite the non-orthodox behaviour of recalcitrant and intermediate seeds— and seeds of the many species that may fall somewhere between these two categories (Berjak and Pammenter 1994)— it is essential that storage strategies be developed, albeit that only short-term conservation for planting programmes and germplasm exchange be facilitated. The immediate problem is that recalcitrant seeds cannot be dehydrated to any water content which would allow low temperature–low RH storage, and it is likely that intermediate seeds of many species too could not withstand the conditions developed to optimize storage longevity for orthodox types.

In fact, overcoming the problems associated with conservation of non-orthodox seeds has been a much-debated topic for at least two decades (e.g. Chin and Roberts 1980; Ouédraogo *et al.* 1996), but very little progress has been made in terms of improved short-term storage of the intact propagules.

As regards recalcitrant seeds, the only conservation strategy for the intact propagules involves their storage at high water contents (essentially undiminished from those characteristic of the newly shed/harvested condition), at the minimum temperature tolerated. In the case of many tropical species, however, these minima must be relatively high, as the seeds are chilling-sensitive. Even for seeds of temperate recalcitrant species, as the tissues are highly hydrated— and continue to be metabolic— storage at sub-zero

temperatures is precluded. Low-temperature storage is also not an option for chilling-sensitive seeds showing intermediate post-harvest behaviour.

The very conditions necessary for viability retention of recalcitrant, and probably most other non-orthodox seeds, are also those that facilitate fungal proliferation (Berjak 1996). Furthermore, any manipulations of such seeds, for example, attempting to lower the water content of recalcitrant types to prevent ongoing events of germinative metabolism, may well prove sufficiently stressful (Drew *et al.* 2000) to exacerbate the deleterious effects of seed-associated micro-organisms, particularly fungi. The same is likely to be the case for dehydrated intermediate seeds (Berjak 1996).

During short-term storage of whole non-orthodox seeds, the proliferation of micro-organisms, particularly of fungi, must be curtailed. It is, of course, desirable that microbial propagules be eliminated completely from the seeds prior to storage, but this is extremely difficult to achieve. Long-term conservation of the genetic resources of species producing non-orthodox seeds is likely to be achieved only by cryostorage of suitably small explants, such as excised zygotic axes (Engelmann 1999). These must harbour no microbial propagules—particularly as all require a period *in vitro* following retrieval from cryostorage, during which any associated fungal or bacterial propagules will flourish. However, the advantage when working with isolated axes, is that these structures which are usually tightly enclosed by the surrounding tissues in the intact seed, are able to be surface-sterilized after excision.

3 The nature of fungi on forest tree seeds

One of the main features of forest tree seeds is their great diversity in size, shape and texture. The size and texture of tree seeds range from small and hard, as the seeds of *Eucalyptus* spp., to the relatively large and fleshy acorns of some *Quercus* spp. or hard walnuts of some *Juglans* spp. The longevity of tree seeds varies from a few days to many years.

The main effects of seed-transmitted fungi are the diseases they cause, and to some extent also the reduced seed viability. However, they rarely destroy the seeds completely. Examples are *Sphaeropsis sapinea*, causing *Diplodia* shoot blight of pines and other conifers, *Sirococcus conigenus*, causing *Sirococcus* blight of pines and other conifers, *Botryodiplodia theobromae*, causing rots in a wide host range and many *Fusarium* spp. causing damping-off of seedlings.

Seed-borne micro-organisms may reduce germination and seed longevity in storage of all types of seeds. When seeds are moved internationally, pathogens may become a quarantine concern and jeopardize seed trade and germplasm exchange. The micro-organisms that are mostly associated with tree seeds are fungi, bacteria and, to a lesser extent, viruses. A comprehensive list was published by Mittal *et al.* (1990). Phytoplasmas, which are known to cause a number of so-called little leaf or witches' broom diseases, are not seed-borne due to the nature of their transmission (phloem-limited, and no connection from phloem to seeds). It is important to distinguish between seed-borne micro-organisms and seed-transmitted micro-organisms. The term seed-borne describes the state of any micro-organism being carried with, on or in the seed. The term seed-transmitted includes the act of infection of the seedlings from seed-borne inoculum. Thus seed-borne micro-organisms include the pathogens causing plant diseases, the so-called 'field fungi' as well as the so-called 'storage fungi'.

Examples for typical 'storage fungi' include *Penicillium* spp., *Aspergillus* spp. and *Caloscypha fulgens*, the seed or cold fungus that kills pine seeds under cool conditions. Their main effect is to reduce seed viability, under certain conditions to even kill the seeds. Poor germination of seedlots can often be attributed to contamination with micro-organisms (e.g. Mwanza and Kellas 1987; Sutherland *et al.* 1987; Huang and Kuhlman 1990). Micro-organisms in general thrive under conditions of high moisture and a temperature range between 20 and 25°C. These are also the conditions that ensure survival of recalcitrant seeds. Although few

systematic surveys of the contamination of recalcitrant seeds with micro-organisms have been conducted (e.g. Mittal and Sharma 1983; Mycock and Berjak 1990; Pongpanich 1990), it appears that they suffer more from effects of micro-organisms on seed quality than orthodox seeds (Berjak 1996). In addition, recalcitrant seeds are more sensitive to common seed treatment with heat or fungicides. Recalcitrant seeds are often conserved *in vitro*, where particularly the storage fungi may cause problems by contaminating the media.

There is a need to conduct more survey work on pathogens of recalcitrant tree seeds, both with regard to quality aspects and with regard to phytosanitary issues. It is very important to keep these two aspects separate in the evaluation of research results. Pest Risk Analysis (FAO 1996) can help in identifying pests of quarantine concern and in suggesting management options. Treatment techniques (chemical, biological or physical) that do not affect seed viability need to be identified.

The majority of pathogens associated with forest tree seeds are fungi, producing only or predominantly the asexual (anamorph) stage (Deuteromycetes). There are exceptions though, as the oomycetes (Oomycota) or water moulds, which can be seed-borne on hardwood seeds, e.g. *Phytophthora cactorum* on beechnuts (Prochazkova and Jancarek 1991). Except for *Rhizoctonia* spp. that are sometimes seed-borne and may have a basidium-producing (Basidiomycotina) sexual stage, the reproductive spores or structures of other Basidiomycotina, are rarely found on tree seeds, and even when present, they are of no consequence. For example, *Heterobasidion annosum*, which causes root rots of forest trees, has been isolated from *Abies* sp. seeds (Mittal *et al.* 1990). However, there is no evidence of this or similar fungi affecting seeds or serving as inoculum to cause root rots. Although rust fungi, which are also Basidiomycotina, frequently attack conifer cones, seeds from diseased cones do not carry the pathogen.

There are many mechanisms by which tree seeds acquire pathogens. Indeed, both the occurrence and severity of many seed-borne fungi, is often traced to the mishandling of fruits, cones or seeds. A major factor in the acquisition of seed-borne fungi is the contamination of fruits or cones with soil, as often occurs when they are collected from the forest floor, the ground beneath seed orchard trees, or when forest trees are felled to facilitate fruit or cone picking. Beechnuts (*Fagus* spp.), for example, collected from the forest floor may be infested with *Phytophthora cactorum* (Prochazkova and Jancarik 1991). Plastic netting can help reduce infection (Fig. 1). Improper handling of

cones or fruits may also contribute to the occurrence of seed-borne fungi, when moulds build up on wet conifer cones stored for prolonged periods following collection. Storing cones wet can also increase temperature within the collection bags, further enhancing moulding. The inclusion of old, infested cones or fruits in current year's collections, can be a source of seed-borne pathogens. *Sirococcus* blight of conifers, caused by *S. conigenus*, is a classic example. The pathogen occurs on old, pathogen-infested conifer cones, especially of spruces, *Picea* spp. Seedlots become affected when such cones, containing infected seeds, are included in collections of current year, disease-free cones. Another source of contamination results from improperly cleaned seedlots containing bits of pathogen-infested needles, leaves, cones or other debris and infected seeds.

Seeds often carry fungi that are considered as saprophytes or weak pathogens, e.g. species of *Penicillium* or *Aspergillus*. Such so called 'storage fungi' are common on stored, but not fresh, seeds (Prochazkova, pers. comm.). This observation invariably leads to the question of what role these fungi play. Are they harmful or do they simply colonize weakened seeds? For example, seeds may be weakened by numerous factors, including long-term or improper storage, or high processing temperatures that are sometimes required for drying or opening cones. Seeds harbouring such fungi invariably germinate poorly or slowly, but it is hard to define the

Fig. 1. Plastic netting suspended above the floor in a beech forest to prevent infection of beechnuts by duff-inhabiting, pathogenic fungi. (Dr Eva Palatova, Mendel University of Agriculture and Forestry, Brno, Czech Republic)

cause and effect. Not all seed-borne fungi are detrimental and sometimes they may be beneficial. For instance, *Trichoderma harzianum* is sold commercially as seed dressing to protect seeds and seedlings from damping-off and root rots. However, under optimal conditions even *Trichoderma* species may be pathogenic (Vaartaja 1957).

Seed analysts are interested in detecting and defining the role of seed-borne pathogens but, except for seeds destined for export or import, it may be impractical to assay all seed lots. The following problems should be avoided when seeds are selected for storage:

- Moulds on the fruits or cones from which the seeds originated.
- Moulds on the seeds during germination tests or following moist treatments such as stratification.
- Presence of pathogenic fungus structures such as sclerotia on or within seeds.
- A high percentage of the seeds fail to germinate and often their contents are rotted.
- Fruits or cones originate from forest stands, seed orchards or mother trees with potential seed-borne diseases, such as *Sirococcus* blight on pines and spruces or pitch canker, caused by *Fusarium subglutinans* f. sp. *pini* on pines.
- Low percentage seed germination, slow germination or both following stratification or other treatments that stress the seeds. Slow germination is an excellent indicator of poor quality seeds.
- Radiographs reveal many seeds that are empty, with cracked seedcoats, abnormal contents or insect damage.
- Fruits or cones harshly treated before or during processing, usually after being subjected to higher that normal temperatures to dry or open them.
- Seeds originated from fruits or cones that had contacted seed orchard soil or the forest floor, e.g. cones collected from squirrel caches.
- Seeds originated from collections containing old or insect-damaged fruits or cones.
- Seed lots contain many immature seeds and much debris.
- Seed lots in which seed viability in long-term storage declines faster than expected.
- Specific fungi that regularly appear on seeds during routine germination tests.
- Disease that consistently appears on specific seedlots of young nursery seedlings.

Specific recommendations for storage of non-orthodox seeds are given in Section 6.4.

4   Some general pre-assay considerations

4.1   Obtaining a representative seed sample

Before assaying for seed-borne pathogens, it is first necessary to obtain a representative seed sample. The most recent edition of the ISTA International Rules for Seed Testing (1999) and Edwards and Wang (1995) cite methods and equipment needed for seed sampling. We recommend that these procedures and recommendations for selection of reliable equipment be followed to guarantee the accuracy of the assays.

4.2   Seed sample size

The number of seeds required for testing is determined according to the expected incidence of the test pathogen in specific seed lots. In general, sample size depends upon the sensitivity required (the more seeds tested the greater the accuracy) and the availability of seed and resources available to process the samples. Determining proper sample size requires that preliminary assays be done to obtain data about the incidence of seed-borne pathogens present in seed lots. Once this information is obtained, sample size can be determined using a binomial distribution (Zar 1984). When using such procedures it is common for sample size to vary by pathogen, tree seed species and accuracy level desired. See Zar (1984) or other statistical texts for help in this area or consult a biometrician whenever there is doubt about the number of seeds needed for an assay. A rule of thumb: if 300 seeds are tested with a reliable method and found healthy, one can be 95% sure that the infection percentage is less than 1%, or: to be 95% sure that the infection in a seed lot is below 0.3%, one has to test 1000 seeds (Diekmann 1993).

4.3   Seed surface sterilization

Another consideration is how to prepare the seeds for assay. Before testing forest seed it can be washed with water or disinfesting chemicals. Exposure to physical agents, such as heat, cold, scarification, or removal of the seed coat, may also be considered as pre-treatments of forest seed. Removing the seedcoat from small seeds is too tedious and time consuming to be justified. However, it may be worthwhile for large seeds such as acorns, particularly if the suspected pathogen is within seeds that have many surface fungi and bacteria. Seedcoats of hardwood seeds, or seedcoats adhering to hardwood germinants, must be removed before assaying for viruses. If the isolation medium is selective for a particular fungus or group of fungi it is

often not necessary to surface-sterilize the seeds as the medium inhibits growth of most contaminants or stimulates the growth of the desired fungus. The most common surface disinfectant is ordinary bleach (NaOCl). While effective, bleach is also residual and so the treated materials must be washed two or three times with sterile water to remove the bleach, which would otherwise inhibit growth of the fungus. We have used 3–30% hydrogen peroxide for surface-sterilizing tree seeds (Sutherland *et al.* 1987). It works well and does not have to be washed off the seeds, but it is very corrosive and must be handled with extreme caution. Handling samples of small seeds is facilitated by using small, cylindrical-shaped (e.g. 5 cm long × 2 cm diameter) 'baskets' made from plastic window screen. The open end of the basket is plugged with a rubber stopper to keep the seeds in the basket that is moved in and out of surface disinfectants or washing water with laboratory tweezers.

4.4   Incubation materials and testing conditions

The protocol dictates the type of apparatus to be used for incubating seeds or seedlings. Most assays in which small to medium-sized seeds are incubated on blotter paper or agar media are done in 90–100 mm diameter Petri plates. Large seeds require bigger containers. Ideally the incubation temperature should be optimum for growth of the target pathogen, but not for other fungi and bacteria. As fruiting bodies and spores are a prerequisite for fungus identification, sometimes methods to promote fungus sporulation may be required. These could be incubation of cultures in ambient daylight or under near ultraviolet (NUV) light ('black light'). Details are given for the respective species.

4.5   Choice of detection methods

The method to be used depends on the target pathogen(s), the purpose of testing, the accuracy level desired and cost. For the detection of ilarviruses and cucumoviruses, the immuno-enzymatic ELISA test is recommended, whereas the immuno-capture-reverse transcriptase polymerase chain reaction (IC-RT-PCR) is used for detecting a nepovirus. The dot blot nucleic acid hybridization technique can be used for detecting tobamoviruses in oaks and maples.

Reeves (1995) reviewed various immunological and nucleic acid methods for detecting seed-borne fungi, bacteria and viruses. As Maude (1996) points out, these techniques are used for detecting fungi in soil and diseased plants, but seldom used in assays for seed-borne fungi. This is mainly because existing tests for detecting

fungi on seeds, including blotter tests and use of selective media, are fairly effective and cheaper to develop and use. However, this does not preclude the need for developing molecular assays for fungi and other pathogens on tree seeds. Simple and reliable procedures are needed, especially for testing seed health of tree species which are widely used in large scale re- and afforestation programmes (Mohanan and Sharma 1991). Already some progress has been made in this direction with the development of monoclonal antibody protocols for detecting *Sirococcus conigenus* on spruce seeds (Mitchell and Sutherland 1986; Mitchell 1988). One of the main advantages of molecular techniques is that they are much more sensitive than existing procedures. Consequently, extremely low levels of seed-borne pathogens can be detected with these advanced techniques, which is particularly important to certify seed for export, or even for long-distance movement within countries, to minimize the risk of disseminating seed-borne pathogens.

5 General protocols for detecting fungi

5.1 Examination of seeds

Before proceeding to more elaborate procedures, first examine dry seeds using a magnifying lens or stereomicroscope. Seeds that are mouldy (Fig. 2), cracked or broken (Fig. 3), or which

Fig. 2. Mouldy seeds of Douglas-fir. (Dr J. Sutherland, Victoria, BC, Canada)

Fig. 3. Acorns with cracked seedcoats, showing black mycelium of *Ciboria batachiana* infection. (Dr Zdenka Prochazkova, Forestry and Game Management Institute, Research Station Uherske Hradiste, Czech Republic)

Fig. 4. Insect-damaged (hole) Douglas-fir seeds. (Dr J. Sutherland, Victoria, BC, Canada)
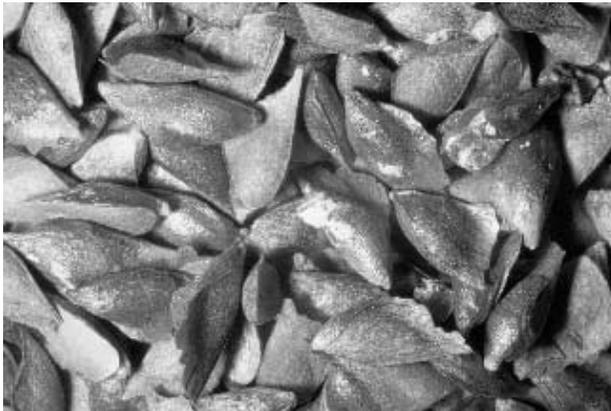
Fig. 5. Resin on Douglas-fir seeds indicates high temperatures were used during cone or seed processing. (Dr J. Sutherland, Victoria, BC, Canada)

have insect damage (Fig. 4) are easily detected. Poor quality seed lots may contain small or abnormal seed. Resin drops (Fig. 5) on conifer seeds indicate the cones or seeds were subjected to excessively high temperatures during processing. If so, the seeds may be weakened and thus susceptible to saprophytes and opportunistic pathogens and not suitable for long-term storage in genebanks. Bits of pathogen-infested needles and leaves and other debris (Figs 6 and 7) can also be seen, as can sclerotia (Fig. 8) and other fungal structures. Poor quality seedlots may contain many small or abnormally shaped seeds. Radiographs reveal many of the same problems and, in addition, help to detect minute cracks in seedcoats, internal insects or abnormal contents of pathogen-infected seeds which would otherwise go undetected. Poor quality seed should be discarded.

Fig. 6. Douglas-fir seeds with debris, a possible source of pathogenic fungi. (Dr J. Sutherland, Victoria, BC, Canada)
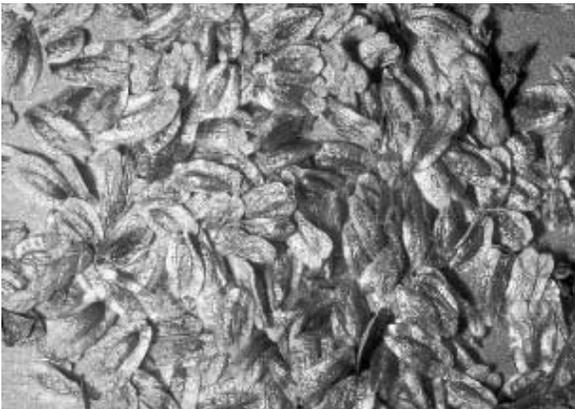
Fig. 7. Western redcedar, *Thuja plicata,* seeds with needle debris (green), a possible source of pathogenic fungi. (Dr J. Sutherland, Victoria, BC, Canada)
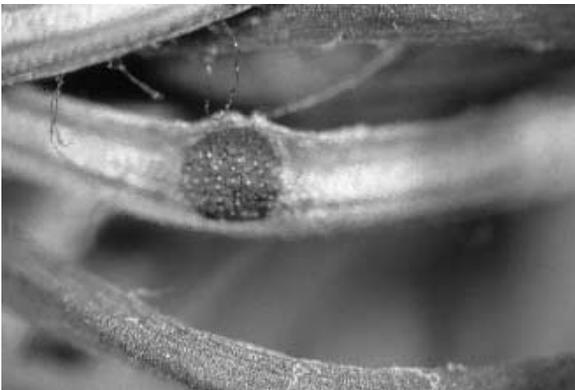
Fig. 8. Round, black sclerotia of the fungus *Botrytis cinerea.* (Dr J. Sutherland, Victoria, BC, Canada)

5.2 Examining seed washings

Recommended for detecting surface-contaminating fungus spores on seeds

The seeds are placed in a flask or other container with water and shaken. For a quantitative test, seed weight and water volume should be known, e.g. 50 g seeds and 50 ml water. Spore counts with the help of a haemocytometer can be calculated as number of spores per millilitre of water, which then equals the number of spores per gram of seed. Adding a drop or two of detergent helps dislodge spores from seeds, but it may create foaming problems. If a low spore load is expected, the seed washings should be centrifuged and the sediment examined by compound microscope for spores.

Yuan *et al.* (1990) used 50 seeds of *Acacia* spp., 200 mg of *Casuarina* spp. and 100 mg of *Eucalyptus* spp. seeds each in 10 ml of water shaken on a 'wrist action shaker' for 10 min. Suspensions of particulate matter were decanted from the seed washings and centrifuged 5 min at 5000 rpm. A compound microscope and haemocytometer were used to examine the sediment in the tubes for spores. Using this procedure they detected *Pestalotiopsis* sp. and *Ulocladium* sp. in seed washings of all three tree genera and Phoma sp. was found in washings from *Casuarina cunninghamiana* and several *Eucalyptus* species. Other fungi detected in washings (tree seed species not given) were *Curvularia lunata*, *Drechslera spicifera* and *Penicillium* sp.

5.3 Detecting fungi during routine seed germination tests

With valuable germplasm, this method helps reduce the number of test seeds. Germinate the seeds according to recognized protocols, usually the ISTA rules (1999). During or at the end of the test, remove and identify the fungi that grow on the seeds or germinants. Transfer those that do not sporulate to PDA, malt agar or other culture media to induce sporulation. However, these recommendations vary according to the test species. For instance, seed may be germinated on top of, or between, paper, in sand, or seed may be soaked in concentrated phosphoric acid or water. Some tree seed requires 14–70 days of incubation before germination can be evaluated.

A major advantage of this procedure is that it is carried out concurrently with routine germination tests. Thus, except for microscopes and references needed to identify the fungi, other equipment and resource requirements are minimal. Other advantages are that many of the fungi sporulate on the seeds or

germinants, and so it is usually not necessary to subculture them on agar or other culture media to induce sporulation. It is also easy to relate and quantify fungus occurrence and abundance to diseases such as seed decay and radicle rot. Some disadvantages are that fast-growing, saprophytic fungi may obscure pathogen growth and the test is not specific for detecting one or a group of pathogens.

Prochazkova and Jancarik (1991) used this technique to identify 141 fungi in almost 6000 conifer seedlots and 170 fungi in over 2200 broadleaf seedlots. The conifer seeds were *Abies alba*, *A. concolor* and *A. grandis*, *Larix decidua*, *Pseudotsuga menziesii*, *Picea abies*, *P. glauca*, *P. omorika*, *P. pungens* and *P. sitchensis* and *Pinus sylvestris*, *P. nigra*, *P. strobus*, *P. mugo* var. *mughus*, *P. mugo* var. *uncinata*, *P. cembra*, and *P. contorta*. Some of the pathogenic fungi isolated were *Botrytis* species from species of *Picea*, *Pinus* and *Larix*, *Fusarium* spp. from species of *Pseudotsuga*, *Pinus* and *Larix* and *Verticillium* spp. from *Pinus* and *Larix* spp. The hardwood seeds assayed were *Alnus glutinosa* and *A. viridis*, *Betula verrucosa*, *Carpinus betulus*, *Fagus sylvatica*, *Fraxinus excelsior*, *F. americana* and *F. angustifolia*, *Sorbus aucuparia*, *Tilia cordata* and *T. platyphyllos* and *Ulmus glabra*. Among the pathogenic fungi obtained were *Ciboria alni*, *C. batschiana* and *C. betulae* from the seeds of species of *Alnus* and *Betula* (<1% of the seedlots tested), respectively. *Fusarium* spp. were detected in all the hardwood seedlots, especially *Fraxinus* sp. seeds where >50% of the lots were infested. *Rhizoctonia solani* was obtained from <1% of the beechnut seedlots. The grey mould pathogen, *Botrytis cinerea*, was found in over two-thirds of the *Carpinus betulis* seedlots. *Trichoderma viride*, which is often associated with poor quality seeds, occurred on over 40% of the *Pseudotsuga*, *Larix* and *Carpinus* seedlots, 30% of the *Tilia* lots, and 15–30% of the lots of *Acer*, *Quercus* and *Sorbus*.

Sometimes pathogens may be detected in tests that are made in conjunction with routine germination tests. For example, cross or longitudinal sections of seeds that germinate poorly are often cut with a razor or scalpel blade to determine embryo development and size. Internal seed decay and presence of moulds can often be seen in such sections

5.4   Blotter test

Recommended for detecting a wide variety of fungi in or on hardwood and conifer seeds.
Place seeds on water-soaked blotter paper in sterilized Petri dishes or other such containers. Tap water can be used as long as it is boiled for 10 min. Sterile water safeguards against contamination by water-borne fungi. Incubate the seeds at 20–25°C, or temperatures favouring

Fig. 9. Seed-borne *Sirococcus conigenus* sporulating on a killed, spruce germinant. (Dr J. Sutherland, Victoria, BC, Canada)

the suspected pathogen, for one to two weeks, or until fungi develop. They can then be identified using the fruiting bodies or spores on the paper, seeds or germinants (Fig. 9), or removed and plated onto a culture medium to induce sporulation. The principle here is that seeds are kept in a humid environment favouring fungus development. The technique is thus similar to that of detecting pathogens during routine seed germination tests and so it is not surprising that the two assays detect many of the same fungi. The major advantage of the blotter test is that it more flexible, e.g. it can be used to assay one to several seedlots using incubation temperatures and lighting regimes that favour fungus detection over seed germination. Another advantage is that it is possible to relate pathogen occurrence and abundance to seed decay and other damage. Sometimes agricultural seeds are killed or weakened by freezing or herbicides, to enhance pathogen development, before being placed on the blotter papers (Maude 1996). Working with both non-and surface-sterilized seeds Sharma and Mohamed Ali (1997) used the blotter technique to isolate a variety of non-pathogenic, potentially pathogenic and pathogenic fungi, e.g. *Fusarium solani*, *F. moniliforme* and *Botryodiplodia theobromae*, from seeds of the tropical hardwoods *Lagerstroemia microcarpa* and *Pterocarpus marsupium*. As in this case seeds may be surface-sterilized before being assayed or the seedcoat can be removed from large seeds with profuse surface contamination. This technique is easy to use and requires minimal equipment; however, rapidly growing saprophytes or weak pathogens such as species of *Penicillium*, *Trichoderma* or *Aspergillus* may overgrow certain pathogens. Also, pathogens such as *Phytophthora* that may lack conspicuous spores and vegetative growth can go undetected.

6 Specific protocols for detecting fungi in non-orthodox seeds

6.1 Fungal activity during recalcitrant seed storage

An in-depth study on the effects of fungi during hydrated storage of *Avicennia marina* has proved very revealing of the significant role of mycoflora in promoting deterioration of recalcitrant seeds (Calistru *et al.* 2000). In that study, after pericarp removal, hand-harvested seeds were treated initially and periodically during hydrated storage by aerosol spraying with a relatively effective fungicide, Previcur N (see below for details). The peculiar morphology of *A. marina* seeds (see Farrant *et al.* 1993 for diagrammatic details) allows fungicide, applied as a fine spray, access to the inner cotyledonary surfaces as well as to the exterior of much of the embryonic axis. The results showed unequivocally that this treatment, which continued to curtail fungal activity, extended the storage lifespan of these 'clean' seeds by almost 50% compared with previous studies in which no equivalent measures were taken (e.g. Berjak *et al.* 1989). In the investigations of Calistru *et al.* (2000), seeds that had been initially treated with the fungicide, but then inoculated with *Fusarium moniliforme*, were all dead within a third of the time for which the 'clean' seeds remained vigorous and showed high viability. In the experimentally infected seeds, rapid fungal proliferation occurred, and was accompanied by profound deterioration of both cotyledonary and embryonic axis cells. What was a highly significant finding though, was that if the seeds had been stored 'clean' for a few days prior to being experimentally infected with *F. moniliforme*, then they were considerably more resilient to fungal depredation, arguing for the development of active defence mechanisms as germinative metabolism progressed under hydrated conditions. This response, however, postponed rather than prevented the seemingly inevitable fungal degradation and viability loss of the stored seeds.

The change in susceptibility of the seeds to fungal attack underscores the importance of developmental stage in these actively metabolic plant propagatory units. Even in the 'clean' seeds though, inherent infection by *F. moniliforme* was not completely eliminated, becoming apparent in localized association with only cotyledonary surfaces, about half way through the experimental storage period although there was no vigorous fungal proliferation. This was the situation in 30% of the 'clean' seed population by the end of the storage period when viability (perhaps, or perhaps not, coincidentally) was 70%. Electron microscopical studies showed that cotyledonary cells contiguous with the fungal mycelium were

extensively degraded but, significantly, cells of the embryonic axis with which no mycelium was associated also showed marked deteriorative changes.

This study confirms the premise (Berjak 1996) that active fungal metabolism during hydrated storage of recalcitrant seeds would impose significant limitations—not only on the total (storage) lifespan—but also on seed vigour, and thus quality. The findings underscore the necessity of finding whatever solutions may be possible, to curtail the incidence—or ideally, to eliminate—the associated fungi from recalcitrant, and all other non-orthodox seeds, prior to storage.

6.2 Fungus incidence in non-orthodox seeds

Fungal propagules may gain access to the seed tissues at any time from flowering to the post-shedding phase. Recalcitrant seeds may be internally infected by fungi *ab initio* by systemic transmission via the parent plant, as has been shown for developing maize caryopses, which are orthodox (Mycock and Berjak 1992; Kabeere *et al.* 1997), or through the stigma-style continuum during flowering (Marsh and Payne 1984). The problem with infection that has originated in these ways during seed development, is that the mycelium has the opportunity to become established deep within the tissyitself—and is consequently very difficult (if not impossible) to eradicate. As is the case for orthodox seeds, insects may both cause damage and act directly as vectors for fungal propagules during seed development. Recalcitrant (and probably all non-orthodox) seeds offer a further advantage to opportunistic invading fungi, in that being shed at high water content, they are very prone to contamination once on the ground (see below) or in storage containers. If the seeds are collected soon after shedding, however, such fungal propagules may be only peripherally located and easy to eradicate if prompt action is taken. Additionally, of course, the storage containers and all devices used to elevate the seeds within them, must be rigorously sterilized.

In Durban, South Africa, scientists have worked with non-orthodox seeds of a wide variety of species and, although the proportion harbouring fungi and/or bacteria varied from one batch to another, some contamination was invariable. In general, fungi were the major contaminants of recalcitrant seeds of tropical, sub-tropical and temperate southern African origin but frequently bacteria occurred as co-contaminants (Mycock and Berjak 1990). These findings have been consistent for tropical and temperate species since then, and we have become particularly aware of the problem since embarking on cryopreservation of embryonic axes

excised from a variety of recalcitrant seed species (Berjak *et al.* 1999a,b). Our experience with intermediate seeds has been limited, but in the case of extensive trials with coffee seeds where fungi could be controlled, bacteria posed an almost intractable problem (unpublished data). It should be noted though that seed-associated micro-organisms are not confined to recalcitrant and other non-orthodox types originating only in the tropical to warm temperate zones (see below). It is possible, however, that the spectrum of fungal species may differ with seed provenance where the geographical localities are widely separated (Table 1). As an example (although specific relationships among seed and fungal species cannot be ruled out as the present state of knowledge is scanty), it seems that recalcitrant seeds harvested in 1990 in southern Africa harboured a basically common spectrum of fungi (Mycock and Berjak 1990), which was essentially different from that associated with seeds collected in the Asia-Pacific region (Pongpanich 1990). In a few instances, particular fungal genera have been isolated from seeds of both cool temperate regions and widely separated tropical/sub-tropical zones (Table 1). This is the case for *Phomopsis* spp., which were associated with a low percentage of acorns (*Quercus* spp., Kehr and Schroeder 1996); some species of the Dipterocarpaceae (Pongpanich 1990); *Trichilia dregeana* (Meliaceae, our unpublished data); *Hevea brasiliensis* (Singh and Singh 1990); as well as with neem (*Azadirachta indica*, Sateesh and Shankara Bhat 1999). However, although identifications to the fungus species level were not presented for all the seeds from which *Phomopsis* was isolated, it is likely that these were different: for example, the species isolated from seeds of commercial rubber and of neem, were *P. heveae* and *P. azadirachtae,* respectively. In contrast, while *Phytophthora* spp. have not been generally recorded as being associated with recalcitrant seeds, this genus has been shown to be seed-transmitted in Ghana, where it poses a serious threat to *Theobroma cacao* causing black pod, which is a widespread and destructive disease of cocoa (Kumi *et al.* 1996).

In the southern African context, Mycock and Berjak (1990) investigated the fungal status of newly harvested recalcitrant seeds of seven unrelated species, ranging in provenance from sub-tropical salt-water estuaries to warm-temperate montane areas. These were: *Avicennia marina, Castanospermum australe, Litchi chinensis, Podocarpus henkelii* (a gymnosperm), *Landolpia kirkii, Scadoxus membranaceus* and *Camellia sinensis*. The findings were that *Fusarium* spp. were present on, or in, the tissues in all but one (*L. kirkii*, a latex-producing species possessed also of apocyanaceous alkaloids) and that species of *Alternaria*, *Cladosporium*, *Aspergillus* and *Penicillium* with *Fusarium*

spp. dominated the spectrum of fungi associated with the fresh seeds. Although, unfortunately, in that study fungi were generally not identified to species level, it was unequivocally established that none was of the xerotolerant group collectively described as the seed storage fungi (e.g. Christensen and Kaufmann 1974). Reference to Table 1 shows that the only storage fungi that have been recorded among the many major isolates, are *Aspergillus flavus* group species in association with seeds of the Dipterocarpaceae (Mittal and Sharma 1982; Pongpanich 1990) and those of *Hevea brasiliensis* (Singh and Singh 1990).

The relative absence of demonstrable storage fungi in association with recalcitrant seeds is not surprising. This ecological grouping comprises xerotolerant species (mainly of *Aspergillus* and *Penicillium*) which generally become apparent on, and in, the tissues of air-dry orthodox seeds in storage (Christensen and Kaufmann 1974), when the competition imposed by the so-called field fungi is curtailed by the low water activity and osmotic challenges of seeds stored at low RH. It is probable though that in intermediate seeds dehydrated to relatively low water contents xerotolerant storage fungal species may manifest themselves, provided that their propagules are present intra-seminally, or in the storage containers. The field fungi, in contrast, require relatively high seed-water contents in order to proliferate— which are precisely those necessary to maintain viability of metabolically active, recalcitrant seeds in storage (Berjak 1996). Whereas species of *Fusarium*, *Cladosporium* and *Alternaria* are considered to be major components of the field fungi on, and in, developing and newly harvested orthodox seeds, this does not preclude others. The essential differences between orthodox seeds— which become increasingly inert— and recalcitrant types, which continue to be actively metabolic, are basic to the spectrum of seed-associated fungi that will be manifested.

In fact, unless they are dormant, recalcitrant seeds might be described as seeds in name only, and are actually far more like seedlings once they have been shed. In many cases, the metabolism associated with their development graduates without any obvious marker event into that of germination (Berjak *et al.* 1989; Farrant *et al.* 1989; Pammenter *et al.* 1994). As a consequence, it may be reasonable to suppose that these propagatory units would be open to invasion by a broad spectrum of fungal species, and not only those classically considered to be field fungi. This is borne out by a consideration of the range comprising the mycoflora found to be associated with some of the few species of recalcitrant seeds that have been examined: the spectrum of fungi is, in fact, considerably more extensive than those listed as major isolates in Table 1.

A further complication is that whatever associated fungi are isolated at any one stage of recalcitrant seed development, the composition of the mycoflora is likely to be different at other stages, both before and after the seeds are shed from the parent plant. This is well illustrated by the information from Kehr and Shroeder (1996) that the most virulent of the primary pathogens, *Ciboria batschiana*, associated with seeds of temperate *Quercus* spp., infects the acorns only after they have been shed onto the soil. Our studies on the stored seeds of four species of tropical or warm temperate origin showed that whereas a mixed mycoflora occurred when the seeds were newly shed, the spectrum narrowed to become dominated by *Fusarium* spp. during storage (Mycock and Berjak 1990). A recent, major survey on fungi associated with developing and mature fruits and seeds and shed seeds of *Trichilia dregeana* has been very revealing of the changing nature of the mycoflora (Table 1). Lack of overt disease symptoms is also no guarantee that the seeds are not infected: the embryos of the majority of seeds of *Theobroma cacao*—97%—were found to be infected by *Phytophthora*, although taken from fruits which were symptomless for black pod (Kumi *et al.* 1996).

In the case of *Trichilia dregeana,* many of the fungi associated with immature or mature fruits were not transmitted to the seeds, although in most cases these were isolated from the interior of the pericarp. These included *Alternaria* spp. and notably *A. alternata*, *Colletotrichum gloeosporioides* group species, *Penicillium aurantiogriseum*, *Pestalotiopsis maculans* and *Rhizopus nigricans*. However, in other cases, fungal species isolated from fruit tissues during seed development, were isolated from the arils of the seeds after they had been shed: the fungi concerned were a *Colletotrichum* species, *Fusarium semitectum*, *F. solani*, *F. subglutinans* and two *Penicillium* species. Seeds of *T. dregeana* are enclosed by a substantial waxy aril which, in the shortest-term, might prove to be a barrier to the ingress of fungal structures to the seed tissues themselves. In the trials with these seeds only one species each of *Phoma* and *Phomopsis,* isolated from the fruit tissues during development, became associated with seed tissues, *sensu stricto*, after shedding. However, what seems to be the case from these studies on fruits and seeds of *T. dregeana* is that the fungi that ultimately became seed-associated were the more serious in terms of their potential pathogenicity.

This would have serious consequences in terms of even short-term storage of these seeds as, if they are kept enclosed for even a few hours after collection, the mycofloral propagules in the aril proliferate vigorously, enveloping the seeds in a mass of mycelium. However, it is vital in terms of *T. dregeana* seed survival, that water

Table 1. Major isolates from fruits and seeds of some recalcitrant species differing widely in provenance

A, aril; C, cotyledon; EA, embryonic axis; P, pericarp; T, testa; ext/int, external/internal surface; imm/mat, immature/mature; pre-/post-ss, before/after seed surface-sterilization; pre-/post-tt, before/after thermotherapy; tiss., within tissues of structure indicated; %, indicated percentage of seeds/structures from which fungus (bacteria) isolated.

	Quercus spp.[1]	Diptero-carpaceae[2]	Shorea robusta[3]	Trichilia dregeana[4]	T. dregeana (shed)[4]	Avicennia marina[5,6]	A. marina stored[5,6]	Hevea brasiliensis[7]
Alternaria spp.				imm:P, ext		P, ext		
A. alternata	P 48% C 20%		2%	imm:P, ext/int				
A. tenuis		6/12 spp.						
Aspergillus spp.						P,C,EA		
A. flavus gp.		5/12 spp.	12%					present
A. niger gp.		12/12 spp.	100%			EA[6]		present
Aureobasidium pullulans	P 5% C 1%							
A. apocryptum	P 2% C 3%							
Botryodiplodia sp.		6/12 spp.						
B. theobromae								>20%–58%
Botrytis cinerea	P 3% C 3%							
Chaetomium spp.		3/12 spp.	2%					
Ciboria batschiana	P <2%‡,†							
Cladosporium spp.	P 4% C 2%	7/12 spp.		mat:P, int; T	C (post-ss)			
C. cladosporioides	P 13% C 12%		6%					
Colletotrichum sp.		3/12 spp.		imm/mat:P, ext/int; mat:A/T	A			
C. gloeosporioides gp.				imm:P, int		C[6]		up to 24%
Curvularia spp.		8/12 spp.						
Discula umbrinella	(10–13%[8])							
Epicoccum nigrum	P 31% C 6%	1/12 spp.						
Fusarium sp.[1]	P 10% C 6%							
Fusarium sp./spp.[5]		6/12 spp.	14%			P,C,EA ext; C,EA tiss[5]	P,C,EA ext, int, tiss[5]	
F. moniliforme		1/12 spp.				EA ext post-ss[6]	C 30% @ 21 d stor.+ fungicide[6]	
F. oxysporum	(recorded[9])							
F. semitectum		1/12 spp.		imm/mat:P, ext;mat:P,ext/int; A/T	A	C 65% post-ss[6]	C 15% @ 14 d stor. + fungicide, 0% @ 21 d 6	up to 15%
F. solani				imm/mat:P, ext/int	A			up to 10%

	Quercus spp.[1]	Diptero-carpaceae[2]	Shorea robusta[3]	Trichilia dregeana[4]	T. dregeana (shed)[4]	Avicennia marina[5,6]	A. marina stored[5,6]	Hevea brasiliensis[7]
F. subglutinans				imm/mat: P,ext	A			
Macrophomina sp.		8/12 spp.						
Penicillium sp./spp. ([6]P. grandicola & P. crustosum)	P 6% C 9%	12/12 spp.				C,EA ext[5,6]		present
P. albicans			16%					
P. aurantiogriseum				imm: P, ext mat: P, int				
P. canadense			80% pre-& post-ss					
P. frequentans			12%					
P. olsoni				mat: P, int; T; C tiss	A			
P.oxalicum & P. cf. brevicompactum				mat: C, int	A			
Pestalotiopsis sp.		4/12 spp.						
Pestalotiopsis maculans				imm: P ext/ T; mat P int				
Phoma spp.	P <2% (?[8])	2/12 spp.		imm: P ext; mat: A	T			present
Phomopsis sp./spp. P. heveae (H. brasiliensis only)	P 5% C 6% post-tt; present[8,9]	2/12 spp.		imm/mat: P ext/int; A	C tiss			present in 9/15 samples, up to 44%
Rhizopus sp.		1/12 spp.		imm/mat: P ext mat: P int				
Rhizopus nigricans			8%					
R. oryzae			36%					
Ulocladium chartarum	P 7% pre-tt, 10% post-tt							
bacteria	P 7% C 10% post-tt					ext/int P/C/EA[5]; ext C[6]		

[1]Kehr and Schroeder (1996); [2]Pongpanich (1990); [3]Mittal and Sharma (1982); [4]our unpublished data; [5]Mycock and Berjak (1990); [6]Calistru et al. (2000); [7]Singh and Singh (1990); [8]Delatour et al. (1980); [9]Murray (1974).
† Ciboria batschiana (see text), an aggresive primary pathogen of Quercus spp., occurs in only isolated instances when acorns are harvested from the tree, but significantly increases after they have been shed (Delatour et al. 1980; Kehr and Schroeder 1996).
‡ Recorded on acorns also by Murray (1974) and on Castanea spp. (Delatour 1978).

content is not lowered (Drew *et al.* 2000) and the intact aril initially serves as a device limiting water loss. However, for storage, the solution to the problem posed by the fungi is for aril removal before the seeds are enclosed at high RH (in fact, in a saturated atmosphere) for storage. This is achieved by soaking the seeds in water for a short time, which softens the aril and allows its removal by gentle rubbing. Thereafter, the seed surfaces have been successfully treated with a Benlate-type fungicide (Fundazol WP, Sanachem, South Africa [active ingredient, benomyl/benzimidazole]) which has ensured their survival for several months under conditions permitting no water loss. However, if the mycelium is internal, then neither this approach— nor any other so far attempted— will facilitate storage of *T. dregeana* seeds, and the same is true for those of all other species. However, it is notable that in no case were any fungi isolated from the axis tissues among the many hundreds of *T. dregeana* seeds used for the trials reported here. This is, however, not always the case: the situation varies from season to season and from one seed-collection locality to another, and there have been instances where *T. dregeana* seeds collected have harboured serious internal fungal infections, which (thus far) we have found to be irremedial and, in the current season, unusually the bacteria have posed a considerable problem after anti-fungal treatments have been used.

6.2.1 Use of fungicides

In cases of established internal fungal infections, the application of systemic types of fungicides may prove effective, considering that recalcitrant (and other non-orthodox) seeds are hydrated when they are shed. Such procedures would necessarily require that the seeds are dried back to the original water contents, as otherwise the problem of their germination in storage would be exacerbated. A very important aspect of drying back, however, is that even if overall seed water content appears to be the same as the initial value, generally that of the embryonic axes is higher than it was before treatment. One must be aware of the fact that for most species, the embryonic axis is only an insignificant fraction of the total seed mass or volume; hence its water content *per se* will make a negligible contribution to that of the whole seed. Yet it is the degree of hydration of the axis that primarily determines the metabolic events that will occur, so elevated axis water contents may mean that germinative metabolism is facilitated at an increased rate, thus limiting even further the storage lifespan of the seeds. Additionally, elevated water content of the axis may well encourage fungal activity, if any inoculum remains in its vicinity. Therefore, assessment of the extent of drying back needs to be

carried out on the separated seed components, until the water content of the embryonic axes approximates to pre-treatment levels. With this proviso in mind, trials with a range of systemic fungicides urgently need to be undertaken for a variety of seed species, to ascertain whether or not this approach could usefully extend their hydrated lifespan, essentially by curbing the proliferation of internally seed-borne fungi.

The essential approach must encompass application of anti-fungal measures when the seeds are newly harvested, and preferably, they should be harvested directly from the parent plant, so as to obviate contact with the reservoir of fungal propagules in the soil. This principle is well-illustrated in the report of Kehr and Schroeder (1996) who showed that although the incidence of fungal colonization by a variety of species (Table 1) increased steadily through the pericarp and cotyledons during maturation on the parent oak tree, it was only after these fruits were shed that the aggressive primary pathogen, *Ciboria batschiana*, infected the acorns.

6.2.2 Removal of seed coverings

The fungicide(s) of choice must be arrived at by identifying the fungi concerned, as well as by testing seed tolerance to its application. As much of the fungal inoculum may be located in the seed coverings, it is expedient to remove these structures, as also suggested by Bonner (1996) but only if this can be done non-injuriously and without curtailing the duration in hydrated storage or causing any damage to the seeds themselves. We have found this to be a beneficial procedure for the recalcitrant seeds of several species. However, the now-exposed seed surfaces must be sterilized, which itself can be a damaging procedure if it is over-rigorous or otherwise too harsh (Berjak *et al.* 1999a, b). Where it has been ascertained that there is no inoculum harboured internally, then surface application of fungicide will suffice, for example, in the case of seeds of *Trichilia dregeana* (see above). In this case, application of a benomyl preparation, Fundazol WP (Sanachem, South Africa), proved effective. However, in experiments with seeds of *Avicennia marina*, dusting of the exposed seed surfaces with this fungicide or Benlate (DuPont, USA) was very damaging. As an alternative, aerosol application of the fungicide, Previcur N as a solution (2.5 g l^{-1}), proved non-injurious and relatively effective (Calistru *et al.* 2000—see above). Previcur (active ingredient propamocarb—HCl [AgrEvo, South Africa]) appears to have a definite potential as a systemic fungicide for the treatment of recalcitrant (and other non-orthodox) seeds prior to storage, and trials on its use alone, or in combination with other fungicides, are

presently being undertaken. As Previcur proved to be fungistatic rather than fungicidal in relation to *Fusarium moniliforme* (Calistru *et al.* 2000), these trials incorporate the use of 'cocktails': in particular, combinations of Previcur and Fundazol or Benlate, and Previcur with Early Impact (Zeneca Agrochemicals, South Africa), are being tested. Early Impact (active ingredients flutriafol [triazole] and carbendazim [benzamidazole]) is being used to replace benomyl fungicides, where these appear to be damaging.

6.2.3 Provision of artificial seed covering

Where the fungi are principally located in the seed coverings, their removal and surface-sterilization of the underlying tissues (as in the case of *Trichilia dregeana*) may be relatively effective in curtailing the problems caused by the mycoflora during hydrated storage. However, it may be advantageous to provide such seeds with an artificial covering, especially one in which a fungicide can be incorporated, or which has natural fungicidal properties. In this regard, the use of alginate gel offers a convenient mode of seed encapsulation. However, it is essential that the degree of hydration of the gel be controlled, so not to afford a source of additional water to the seeds, which would accelerate germinative metabolism in the storage containers.

In Durban, South Africa the research group has access to a crude alginate gel which is custom-made from a brown alga. We are presently experimenting with this gel as an encapsulation medium for de-coated recalcitrant seeds of a variety of species, and have found its use highly effective in significantly extending the storage lifespan of some species. Interestingly, this effect for *Avicennia marina*, could not be correlated with any metabolic parameters of the seeds, but no fungal proliferation occurred during hydrated storage lifespan—which was extended fourfold in comparison with unencapsulated seeds (Motete *et al.* 1997).

6.2.4 Thermotherapy

This is another approach that should be tested for non-orthodox seeds of any species, prior to their hydrated storage. In fact, this should be the first possibility to be assessed, although its success is far from assured in all cases (see below). Thermotherapy, however, has been extremely successful in the treatment of seeds of *Quercus* spp. prior to storage of the acorns. This approach, which was developed by Delatour (1978), involves submerging the acorns in water held at temperatures of around 40°C for 2 h. As discussed above, it is essential that the seeds are then dried back to acceptable storage water contents. Kehr and Schroeder (1996) showed that

many of the fungi associated with *Q. robur* acorns were eliminated by thermotherapy, for example, *Cladosporium cladosporioides*, which originally occurred as a pericarp and cotyledon infection in 13% and 12%, respectively, of the seeds tested. Notably, those authors reported that *Ciboria batschiana*, although its incidence was very low initially (Table 1), was also eliminated. Delatour *et al.* (1980), who stored treated acorns with the further precaution of inclusion of a thiram-based fungicide (TMTD) in the water during thermotherapy (39°C, 8 h), showed zero recurrence of infection by *C. batschiana* after an 8-month period. It is notable that in those studies, *C. batschiana* was isolated from 36% of the acorns prior to treatment. The incidence of other fungi has been found to be significantly diminished by thermotherapy of acorns: for example, Kehr and Schroeder (1996) reported that *Alternaria alternata*, which was isolated at 48% from pericarps and 20% from cotyledons of control *Q. robur*, occurred in 26% and 4% of the pericarp and cotyledonary samples tested, following thermotherapy.

However, thermotherapy is not a 'cure-all' for all seed-associated fungal species: *Penicillium* spp., for example, increased in incidence to 21% and 7% in acorn pericarps and cotyledons after thermotherapy, compared with their isolation from 6% and 9% of these structures, respectively, before the hot-water treatment (Kehr and Schroeder, 1996). It is probable that the *Penicillium* spp. present, which could obviously tolerate the 41°C thermotherapy, could proliferate far more readily in the absence of certain of the fungal species that were eliminated or reduced by the treatment.

In other cases, thermotherapy just cannot be used. Work on seeds of *Avicennia marina*, showed them to be far more sensitive to elevated temperatures than were the fungi that they harboured. The seeds were found to be lethally damaged at temperatures of 45°C, while the virulent strain of *Fusarium moniliforme* that was isolated from them, could withstand temperatures in excess of 60°C (our unpublished data). A vital aspect of thermotherapy trials for non-orthodox seeds therefore, is that at the outset tolerance of the seeds themselves to various temperatures for different periods of time, must be assessed. Additionally, testing the effect of various combinations of temperature and time tolerated by the seeds, on the fungi isolated from those seeds, is ultimately a time-saving and rational preliminary approach.

6.2.5 Other procedures

Are there other treatments that could be used to eliminate or curtail the fungi associated with recalcitrant seeds during hydrated storage? Again, it is impossible to generalize: Kehr and Schroeder (1996)

have discussed the possibility of cold-hardening acorns, so that they will tolerate lower storage temperatures than if untreated. Those authors cite the possibility that *Q. robur* acorns might survive storage at –9°C, while those of *Q. petraea* could survive at –5 to –6°C. Such treatment would go far to minimize fungal effects during seed storage, but it must be borne in mind that *Quercus* spp. are temperate, and are not chilling-sensitive in the first place. It would be surprising if non-orthodox seeds of tropical/sub-tropical species could be so-acclimated.

Another approach suggested as promising for acorns by Kehr and Schroeder (1996) is the use of what has been termed ETS (electron treatment of seeds). While those authors report that this approach has been developed in Germany for control of seed-borne pathogens in cereal seeds, it is noteworthy that its use for improvement of maize seeds was first reported by Pammenter *et al.* in 1974. Those authors, who termed the technique 'cathodic protection' showed a dramatic improvement in vigour and viability retention of the caryopses during storage on a negatively charged conductor, under age-accelerating conditions, but ascribed the benefit to free-radical reducing effects in the seeds themselves. This does not, of course, rule out the possible effectiveness of the treatment by reducing the incidence of seed-associated fungi and their metabolic effects, especially as free-radical generation is considered to be one of the deleterious consequences in the host tissue of fungal metabolism.

It is, however, presently considered unlikely that this type of approach will protect recalcitrant seeds. In the first place, even if electron treatment were to affect peripherally located fungi, in many cases in recalcitrant seeds the fungi occur in the deeper seed tissues. Secondly— and what may be a more important consideration— is the fact that cereal seeds are orthodox and dry, but the associated mycoflora is hydrated and active, which may well be the basis for its destruction by the applied charge, as has been proposed for the success of thermotherapy of superficially wetted maize caryopses (Berjak *et al.* 1992). Recalcitrant seeds, in contrast, are relatively highly hydrated and metabolically active, and it is essential that they remain so. It is thought likely, therefore, that such treatment might be as deleterious to the seeds as it could be to peripherally located mycoflora. However, in the case of non-orthodox seeds that will withstand dehydration— e.g. intermediate seeds— ETS might well be effective, but perhaps only if any inoculum present is peripherally located.

A further approach, mentioned by Kehr and Schroeder (1996) as apparently having a beneficial effect on the elimination of fungi

from acorns, is the use of combined microwave/steam treatment. However, as the basis of microwave activity resides in water (and lipid) heating, one must be critically aware of the possible deleterious effects on the seed tissues. The balance among microwave intensity, the duration of its application and seed water content, is absolutely critical: for example, without the provision of an energy-absorbing buffer, application of microwave therapy to soybeans at the low water content around 0.08 g g^{-1} for less than 30 s, has been found to cause seed bursting (Reddy *et al.* 2000). Recalcitrant seeds are generally large and have water contents anywhere in the range of approximately 0.3 g g^{-1}–0.45 g g^{-1} depending on the species and the developmental status (personal observations). As such, it is difficult to envisage the successful use of microwave energy to eliminate fungi that are located anywhere other than in the peripheral seed tissues. This is because the time taken for the interior of the seed to be sufficiently affected by the microwave energy to eliminate deep-seated inoculum would presumably devastate the peripheral tissues, but more significantly, unless the surface were perforated, the build-up of steam within the seed, would be likely to cause bursting.

Bonner (1996), in discussing possible treatment of non-orthodox seeds against seed-borne pathogens and non-pathogenic micro-organisms, mentions the problem of their internal localization. That author makes the point that, because of the nature of the seeds which is so different from orthodox types, gaseous fumigants are also not an option for recalcitrant seeds.

6.3   Fungus isolation/identification from non-orthodox seeds

In terms of fungal isolations and diagnoses that have been reported, in some instances there is a commonality with the methods reported elsewhere in this publication for fungi associated with orthodox seeds. This is the case for species of e.g. *Fusarium*, *Aspergillus*, *Penicillium*, *Colletotrichum* and *Phytophthora*.

In other cases, specific or general isolation media are described or mentioned. For the mycoflora of acorns, Kehr and Schroeder (1996) used 2% malt agar containing 50 mg l^{-1} streptomycin, with the subculture of developing fungal colonies on malt agar: these were induced to sporulate after growth for two weeks (at room temperature) by maintaining the cultures for a week at 15°C under black light (Philips UVA 40W/08). Delatour *et al.* (1980) mention culturing *Ciboria batschiana* in tubes on malt agar.

Kumi *et al.* (1996) have detailed the medium they used for selective isolation of *Phytophthora* spp. Washed seeds removed from newly

opened cocoa fruits were surface sterilized with 93% alcohol (presumably ethanol) and incubated singly in Petri dishes on the following medium: 23 g l^{-1} Lima Bean agar (Difco Bacto) containing 10 mg each of pimaricin and benomyl, 200 mg vancomycin, 50 mg hymexazol, 60 mg each chloramphenicol and penicillin G. Incubation was for 3–5 days, in 12 h alternating cycles of NUV light and dark. Certain of the isolations were made from separated seed components.

In work on the fungal flora of dipterocarp seeds in general (Pongpanich 1990), *Shorea robusta*, in particular (Mittal and Sharma 1982) and *Hevea brasiliensis* [Euphorbiaceae] (Singh and Singh 1990), aside from using the blotter test, all authors also used PDA plates as the agar method of choice. Isolation of *Phomopsis azadirachtae* and other fungi from neem seeds, Sateesh and Bhat (1999) used a standard agar method based on Czapek–Dox medium.

For recalcitrant seeds of the various species studied in Durban, South Africa, the general approach is to isolate fungi from separated components (coverings, storage tissues [cotyledons/endosperm] and embryonic axes). For all isolations other than those from the outer surfaces of the pericarp, the seeds/seed components are surface-sterilized, generally using 1% sodium hypochlorite with a trace of a wetting agent such as Tween 20 (15 drops l^{-1} sterilant). The duration of surface sterilization varies from 10 to 25 min, depending on the material and the effectiveness of this treatment. Generally PDA is used for the initial isolations, and when necessary for identification, sub-culturing onto other media is carried out.

For fungal isolation from fruit and seed material of *Trichilia dregeana*, initial isolation was in Petri dishes on Czapek–Dox (CDA) medium containing 50 µg ml^{-1} rifampicin. The pH of the medium was adjusted to 6.6 prior to autoclaving, and the filter-sterilized rifampicin added after the autoclaved medium had cooled. Cultures were incubated for 5–10 days in the dark at 25–27°C. The initial isolates were sub-cultured onto CDA to obtain axenic cultures for expert identification. These, in turn, were sub-cultured onto malt extract agar slants, for storage at 4–5°C: stored cultures were sub-cultured every 2–3 months, and re-stored after initial incubation at 25–27°C for 7 days in the dark.

6.4   Recommendations for non-orthodox seeds to be stored

As has emerged from the limited investigations on the fungal problem in non-orthodox seeds generally, and recalcitrant seeds in particular, there is little pattern in the presence of seed-associated

fungi, whether pathogens (*sensu* Plant Pathology) or not. However, irrespective of the pathogenic status of the fungi, the high RH conditions necessary for recalcitrant seed storage demand that the primary approach must be to develop treatments that will ideally eliminate, but at least curtail, the mycoflora. Certainly at present, such treatments will have to be determined empirically, on a seed-species basis—but also taking into account the prevalent fungal species.

Thus, unlike the situation in orthodox seed health testing, it is not so much a matter of what seed-associated fungi are present—and hence of diagnosis—but the imperative of the development of seed treatments, if any reasonable opportunity of short-term seed storage and exchange is envisaged. Hence:

1. the infection status of the seeds needs to be ascertained, on the basis of the separated components (seed coverings, storage tissues, embryonic axes);
2. expert identification of axenic isolates should be made;
3. suitable surface-sterilization procedures need to be established;
4. the effects of removal of the seed coverings must be ascertained;
5. experimentation with artificial seed coverings should be undertaken;
6. extensive experimentation with fungicide treatments is required, particularly concentrated on the possibility of using systemic fungicides;
7. the possible use of thermotherapy requires to be assessed;
8. other treatments should also be entertained;
9. if effective fungicidal treatments can be developed, then it might be necessary (e.g. if *in vitro* culture is to be used, as in the case of cryostorage) to develop suitable anti-bacterial treatments.

7 Isolation of fungi

7.1 Isolating *Fusarium* species

Many pathogenic and non-pathogenic species of *Fusarium* (Deuteromycotina, Hyphomycetes) occur on and in tree seeds. For example, Ciesla *et al.* (1996) reported seven *Fusarium* spp. from eucalyptus (*Eucalyptus* spp.) seeds while Mittal *et al.* (1990) list 29 *Fusarium* spp. or f. sp. on numerous species of tree seeds. In one study alone, Prochazkova and Jancarik (1991) isolated 28 *Fusarium* spp. from seeds of many temperate broadleaf and conifer species. While not all seed-borne *Fusarium* spp. are pathogenic, at least under the conditions tested to date (e.g. Axelrood *et al.* 1995), many cause seed rots, damping-off (Fig. 10), root rots (Fig. 11) and blights in nurseries (e.g. James *et al.* 1991). Others, including the pine pitch canker pathogen *F. subglutinans* f. sp. *pini*, affect older trees (Barrows-Broaddus 1987; Diekmann and Sutherland 1998). While *Fusarium* spp. can be detected on seeds during routine germination or by the blotter test, it is best to isolate the fungus directly from seeds using selective media (Fig. 12). When surface contamination is severe, seeds can be surface-sterilized before plating them onto the medium or the seedcoat can be removed from large seeds. The two most frequently used media for isolating *Fusarium* species are those of Nash and Snyder (1962) and Komada (1975), or modifications of these.
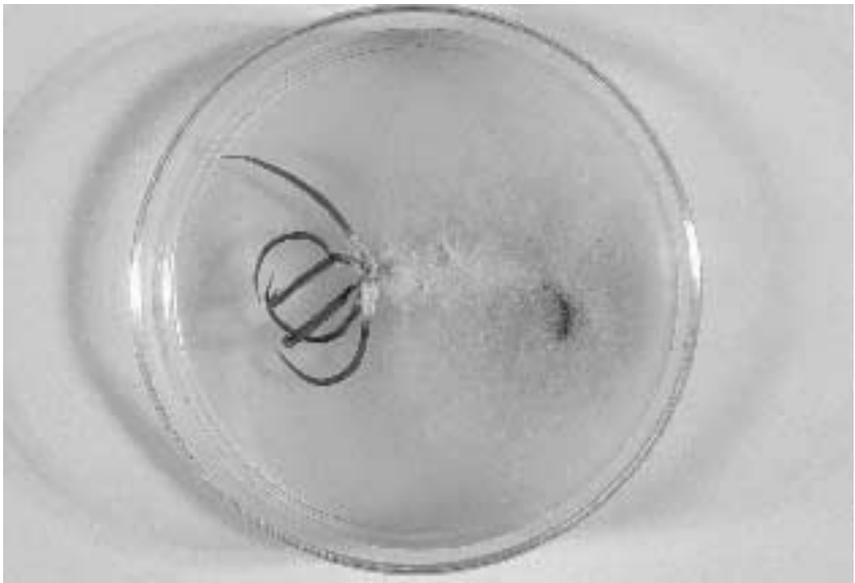
Fig. 10. *Fusarium* sp. growing from a conifer seedling killed by damping-off. (Dr J. Sutherland, Victoria, BC, Canada)

Fig. 11. Douglas-fir seedling killed by *Fusarium* root rot. (Dr J. Sutherland, Victoria, BC, Canada)

Fig. 12. Colonies of *Fusarium* growing from beechnuts plated onto a *Fusarium*-selective medium. (Dr Zdenka Prochazkova, Forestry and Game Management Institute, Research Station Uherske Hradiste, Czech Republic)

The following are the ingredients and procedures for making Nash and Snyder's (1962) medium (modified from original):

Agar ....16 g
Peptone ....12 g
$KHPO_4$ (monobasic) ....800 mg
$MgSO_4$ ....40 mg
Distilled water ....800 ml

Autoclave for 15 min, then immediately add:

PCNB (pentachloronitrobenzene) ....0.6 g[†]
Allow the medium to cool to 50°C, then add:
Neomycin sulphate, 1% solution ....8 ml
Streptomycin sulphate, 5% solution ....8 ml

Incorporate the ingredients into the medium, e.g. using a magnetic stirrer to cool and just before the medium solidifies, dispense into Petri plates.

The ingredients and procedures for Komada's (1975) medium are:

Agar ....15 g
KH_2P_4 (monobasic) ....1 g
KCl ....500 mg
$MgSO_4 \cdot 7H_2O$..500 mg
EDTA ($C_{10}H_{14}N_{20}8Na \cdot 2 H_2O$)...100 mg
D-Galactose ....20 g
L-Asparagine ....2 g
Distilled water ....1000 ml

Autoclave the above mixture for 15 min, allow it to cool to 50°C, before adding:

$Na_2B_4O_7$..1 g
PCNB ....1 g[†]
Bovine ox gall powder ....500 mg
Streptomycin sulphate ....300 mg
Chlortetracycline hydrochloride ....100 mg

Then add sufficient 10% phosphoric acid to take the pH to 4.

Seifert (http://res.agr.ca/brd/fusarium/) gives the recipes for two other *Fusarium* isolation media, i.e. dichloran chloramphenicol peptone agar and peptone PCNB agar. Both are from Burgess *et al.* (1988) and differ slightly from the above recipes. Singh *et al.* (1991) have also formulated a recipe for a *Fusarium* selective medium [see section 5.5.10].

[†]   Note: fungicides in the selective media are given as a.i. (active ingredient, i.e. the amount of active fungicide in the formulated product). For example, a fungicide listed as 80 WP contains 80% active ingredient in a wettable powder formulation; thus 2 g of commercial product would have to be added to obtain 1.6 g of active ingredient.

A 100 mm diameter Petri dish accommodates about 20 medium-sized seeds, e.g. of most conifers, and since these selective media inhibit the growth of many contaminants the seeds can be added to the dishes without following strict aseptic conditions. Incubate the plates at 24–26°C for 7–10 days (James *et al.* 1991) under cool, fluorescent light to induce *Fusarium* sporulation (Komada 1975) which is required for identification.

Based on cultural and spore characteristics, hosts, etc., there are several taxonomic keys for identifying *Fusarium* species, e.g. for those with access to the Internet Seifert's FUSKEY—*Fusarium* Interactive Key (http://res.agr.ca/brd/fusarium/) is available in English and French. Gerlach and Nirenberg (1982) give other keys. Singh *et al.* (1991) give cultural and spore characteristics, and coloured illustrations of colonies, for many species of *Fusarium* from cereal and other seeds, several of which could occur on tree seeds.

7.2   Isolating *Sirococcus* species

The fungi of concern here are *Sirococcus conigenus*, the *Sirococcus* blight pathogen, and *S. clavigignenti-juglandacearum*, which causes *Sirococcus* canker of butternut. *S. conigenus* (syn. *S. strobilinus* and *S. piniperda*) affects several species of conifer, notably *Pinus* spp. (Fig. 13), *Picea* spp. and Douglas-fir (*Pseudotsuga menziesii*).

Fig. 13. *Sirococcus* blight of container-grown, yellow pine, *Pinus ponderosa*, nursery seedlings. Note the disease spreading outward from where it originated on seed-borne inoculum. (Dr J. Sutherland, Victoria, BC, Canada)
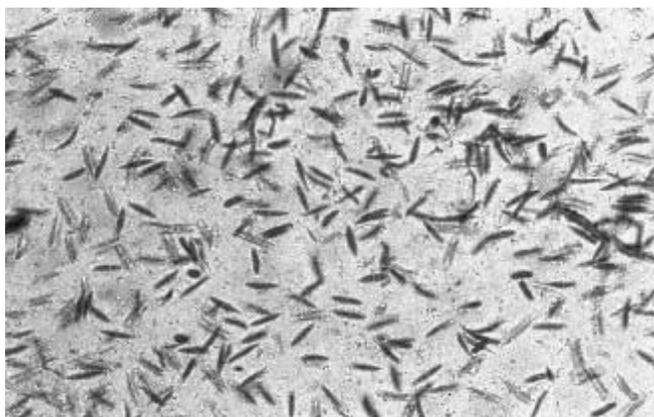
Fig. 14. Spindle-shaped pycnidiospores of *Sirococcus conigenus*. (Dr J. Sutherland, Victoria, BC, Canada)

S. clavigignenti-juglandacearum affects butternut (*Juglans cinerea*) trees in nature and, via artificial inoculation, other *Juglans* species (Sinclair *et al.* 1993). No sexual state is known for either fungus (Deuteromycotina, Coelomycetes) and both fungi are seed-borne, i.e. *S. conigenus* on conifer seeds (Sutherland *et al.* 1987) and *S. clavigignenti-juglandacearum* on butternut (Orchard, 1984; Prey *et al.* 1997). Orchard (1984) demonstrated the seed-borne nature of butternut canker by isolating the pathogen from necrotic tissue near the point of seed attachment of up to 13% of seedlings of infested, butternut seedlots. *S. conigenus* often fruits on germinants (Fig. 9), thus it may also be detected during routine seed testing or by blotter plate testing. However, such tests are less reliable than the one described here since *S. conigenus*, which is often within seeds, is easily overgrown by contaminants.

The following procedures are recommended for detecting *S. conigenus* on conifer seeds and *S. clavigignenti-juglandacearum* on butternut (*Juglans cinerea*) seeds. Surface-sterilize seeds with 30% H_2O_2 for 30 min at room temperature and then plate them, 20–25 per Petri dish, directly onto 2% water agar If H_2O_2 is unavailable, surface-sterilize the seeds with 0.5% NaOCl for 5 min, rinse them twice with sterile, distilled water then plate them onto water agar. Incubate the seeds at 20 to 25°C and every 2–3 days, for up to 2 weeks, use a stereomicroscope to check for *Sirococcus* growing from the seeds or sporulating (Fig. 14) on the germinants. Since incidence of the fungus within infested seedlots is seldom above 3%, test 500–1000 seeds/seedlot to detect the fungus. *S. conigenus* can also be detected in seedlots using a monoclonal antibody protocol (see our discussion on molecular techniques). Sutherland *et al.* (1987) give the characteristics of *S. conigenus* spores and cultures.

Isolations were made by surface-sterilizing diseased tissue in 0.5% NaOCl (time not specified) followed by plating onto PDA. *S. clavigignenti-juglandacearum* was also isolated from aqueous solutions of butternut meats of many of the same seedlots for which seed-borne inoculum of the fungus had been found. Nair *et al.* (1979) and Sinclair *et al.* (1993) give the spore and fruiting body characteristics for identifying this pathogen.

7.3. Isolating the cold or conifer seed fungus

Caloscypha fulgens (Ascomycotina, Pezizales; anamorph *Geniculodendron pyriforme*) is seed-borne and attacks seeds before they germinate in nurseries and forests. The fungus gets its common names because it is seed-borne and it can grow at fairly low temperatures. Seeds are not susceptible to infection once germination begins. Affected seeds are mummified rather than rotted, thus seed lots suspected of being infested should be assayed. Seed lots that germinate poorly following stratification (when the fungus spreads) are prime candidates for assay.

The following procedure is recommended for isolating *Caloscypha fulgens* from conifer seeds, especially of species of *Picea* and *Pinus*. Surface-sterilize seed samples for 30 min in 30% H_2O_2, then plate them 20–25 per 90 or 100 mm Petri dish onto 1.5–2% water agar. Incubate the plates at 15–20°C in either the dark or under no more than 12 h of fluorescent lighting per day. After 10–14 days incubation use a stereomicroscope to look for the distinctive indigo or orange fungus colonies growing from diseased seeds (Fig. 15). Individual hyphae are thick, verrucose
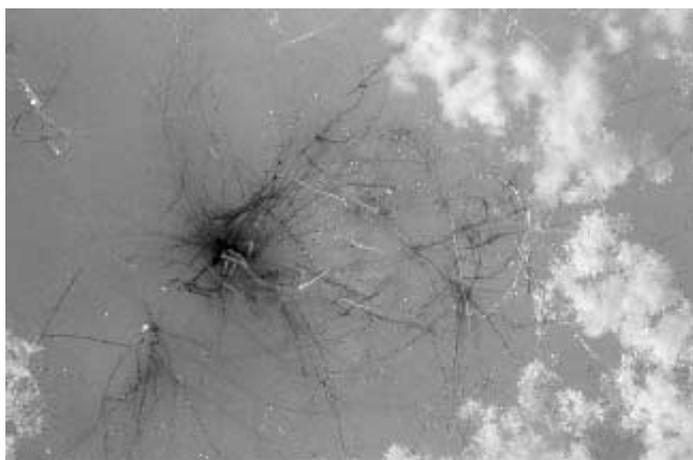
Fig. 15. Indigo-coloured hyphae of the seed or cold fungus growing from a surface-sterilized spruce seed on water agar. (Dr J. Sutherland, Victoria, BC, Canada)

Fig. 16. Thick, verrucose, right-angle branching hyphae of the seed or cold fungus. (Dr J. Sutherland, Victoria, BC, Canada)

and often branch at right-angles (Fig. 16), reminiscent of *Rhizoctonia* spp. The characteristics of the cultures, the asexual spores and the orange ascocarps of the fungus, which are produced on the forest floor soon after spring snow melt, are given by Sutherland *et al.* (1987).

7.4   Isolating *Phytophthora* and *Pythium* species

Whereas species of *Phytophthora* and *Pythium* (Peronosporales) are rather common on broadleaf tree seeds, they are rare on conifer seeds (Mittal *et al.*1990). Perhaps this is because conifer cones are woody and dry at harvest and unsuitable for these fungi, while hardwood fruits that are often succulent or fleshy, or both, are better substrates for *Phytophthora* and *Pythium* species. Infection occurs when fruits contact infested soil, usually in the forest before collection for seed extraction. *Phytophthora* and *Pythium* can be isolated directly from surface-sterilized fruits or seeds, however, since contaminants sometimes overgrow them or inhibit their growth it is best to isolate them using baits or selective media.

A baiting method for isolating *Phytophthora* and *Pythium* from soil or plant material, should work well for seeds, is the apple technique (Campbell 1949).

Use a cork borer to remove a core of tissue from a ripe apple and place the test seeds or other plant tissues into the hole. If the seeds or other test samples are dry then moisten them with sterile, distilled water. Seal the opening of hole with masking tape and incubate the apple at room temperature. When the apple begins to rot around the inoculation core, surface-sterilize it with ethyl alcohol or weak bleach (ca. 5%), remove the skin over the rot, then aseptically remove small segments of apple tissue just ahead of the margin between the rotted and firm tissue. Plate this tissue onto a medium (e.g. PDA) for isolation of *Phytophthora* or *Pythium* spp., or preferably onto one of the selective media given below. Other fruits, including pears, can also be used (Fig. 17).

Fig. 17. Pear baits can be used to isolate *Phytophthora* or *Pythium* from water, soil or seeds. Pear tissue, taken from the margin of the lesions, is plated onto the *Phytophthora*-selective medium in the Petri dish. (Dr J. Sutherland, Victoria, BC, Canada)

Hamm and Hansen (1991) give the recipes for several media for isolating and identifying species of *Phytophthora* and *Pythium* spp. The ingredients and procedures for making two of these follow:

Medium 1. *Corn meal agar with pimiricin (CMP)*
Recommended for isolating and determining the growth rate of Phytophthora *spp. where bacteria are not a problem, and for long-term storage of these fungi.*

Cornmeal ....17 g
Distilled water ....1000 ml
Pimiricin (concentration of active ingredient)[†] ....20 mg
Combine, then autoclave these ingredients for 15 min.

Medium 2. *CMP+ampicillin+rifamycin (CMP+A+R)*
An excellent medium for isolating Phytophthora *and* Pythium *from plant tissues, where bacteria is a problem.*

Corn meal agar ....17 g
Distilled water ....1000 ml
Pimiricin ....20 mg in distilled water
Combine, then autoclave the ingredients for 15 min. Allow the medium to cool to 45°C, then add 250 mg of ampicillin and 10 mg of rifamycin. Prepare new stock solutions for each batch of medium using sterile water for ampicillin and 50% ethanol for rifamycin (store both at 5°C).

Hamm and Hansen (1991) also give the recipes for several media to induce sporulation in *Pythium* and *Phytophthora*, plus a taxonomic key for identifying *Phytophthora* spp. important as forest pathogens. Stamps *et al.* (1990) present a detailed tabular key to *Phytophthora* species and Van Der Plaats-Niterink (1981) gives a taxonomic key for *Pythium* species.

7.5   Isolating *Rhizoctonia* species

Fungi in the genus *Rhizoctonia* are well known, ubiquitous pathogens, causing innumerable diseases on a wide variety of host plants including damping-off and root rots in forest nurseries. *R. solani* is among the most widespread and prevalent species. It is the mycelial, sclerotia-forming state of the fungus *Thanatephorus cucumeris* (Tulasnellales, Ceratobasidiaceae). *R. solani* typifies these fungi that ordinarily produce no spores, have pale to dark brown mycelium, and large-diameter hyphae which branch frequently at right angles. *R. solani* produces black sclerotia, both in nature and in culture, varying in size from spheres less than 1 mm across to crusts several millimetres in diameter (Sinclair *et al.* 1993).

Mittal *et al.* (1990) cite 10 incidences of *Rhizoctonia* on tree seeds, i.e. three for *Rhizoctonia*. sp. and seven for *R. solani.* The records for *Rhizoctonia* sp. are on *Cedrus deodora* in India, *Cupressus sempervirens* in Egypt and *Pinus elliottii* in Taiwan. *R. solani* is reported from a variety of conifer and broadleaf species

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[†]   Also sold as Devoced (Gist-Brocades, King of Persia, Pennsylvania), available as a 50% wettable powder, store the powder in a refrigerated, amber-coloured bottle out of direct light. Always prepare fresh solutions before using.

as for example *Abies* spp. in the USA, *Pinus nigra* in Italy, *Fagus sylvatica* in France and *Araucaria cunninghamii* in Australia.

Usually *Rhizoctonia* species are easily isolated by plating surface-sterilized seeds or tissues onto culture media such as PDA or in the blotter test. However, it is also possible to use baits and selective media for isolation. Chandelier (1994) gives a baiting technique for isolating *R. solani* from soil, which also seems appropriate for isolating the fungus from seeds, although it may be too laborious. The technique consists of soaking 10 mm diameter, filter paper disks in a liquid culture medium, allowing the fungus to colonize the disks, then isolating it from the disks. The ingredients and procedures for making this medium are:

Galactose	20 g
Glycine	2.25 g
KH_2PO_4	1 g
$MgSO_4$	100 mg
$FeSO_4$	5 mg
$ZnSO_4$	5 mg
$MnCl_2$	2 mg
$CuSO_4$	5 mg
Streptomycin sulphate	100 mg
Rose bengal	100 mg
Benomyl	5 mg[+]
Propamocarb	50 mg[+]
Distilled water	1000 ml

Combine the ingredients, autoclave the medium for 30 min, allow it to cool to ca. 50°C, then immerse the disks in the medium for 30 min, shaking it every 5 min. Next drain the disks and air-dry them for 2 h at 50°C. They can be stored up to 2 weeks, preferably in a closed, refrigerated container. Add an appropriate number of disks to a sample of moistened seeds and incubate in the dark for 5 days at 25°C. Then plate out the discs (5–10 per 100 mm diameter Petri dish) onto the following medium:

K_2HPO_4	1 g
$MgSO_4 \cdot 7_2HO$	500 mg
KCl	500 mg
$FeSO_4 \cdot 7H_2O$	10 mg
$CuSO_4$	1.3 mg
$NaNO_3$	200 mg
Agar	15 g
Distilled water	1000 ml

Combine these ingredients, autoclave for 30 min, allow to cool to 45–50°C, then add the following:

Tannic acid ....120 mg
Propamocarb ....800 mg[+]
Streptomycin sulphate ....100 mg
Chlortetracycline...50 mg
Etridiazole...5 mg
Benomyl ....1.3 mg[+]

Dispense the medium to Petri dishes, add the disks, incubate at 25°C and after 2–4 days examine the plates for Rhizoctonia using a stereomicroscope.

Ko and Hora (1971) were among the first to develop a selective media for isolating *R. solani.* Later Gangopadhyay and Grover (1985) improved the efficacy of this medium and although it was developed for isolating *R. solani* from soil it should suffice for isolating this *Rhizoctonia*, and others, from seeds and fruits.

The ingredients and procedures for making this medium are:
K_2PO_4..1g
$MgSO_4 \cdot 7H_2O$ ....500 mg
KCl..500 mg
$FeSO_4 \cdot 7H_2O$ ....10 mg
$NaNO_2$ ....200 mg
Agar...20 g
Distilled water...1000 ml

Mix these ingredients, autoclave the medium, then add:
Gallic acid...400 mg
Fosetyl-aluminium ....250 mg

The latter two are prepared as stock solutions (w/v) using sterile, distilled water.

We recommend that surface disinfected seeds, or portions of fruits, be plated directly onto this medium in Petri dishes. Incubate and examine the plates using a stereomicroscope. Sneh *et al.* (1991) give several other isolation techniques and selective media for isolating various *Rhizoctonia* species, mainly from soil. Chandelier (1994) outlines identification methods for Rhizoctonia species and Sneh *et al.* (1991) provide techniques and taxonomic keys for identifying *Rhizoctonia*.

7.6   Isolating *Cylindrocladium* species

Species of *Cylindrocladium* (Deuteromycotina, Hyphomycetes) cause damping-off, root rots and foliage blight of many species of broadleaf and conifer seedlings. Unidentified species of *Cylindrocladium* have been reported as being seed-borne for *Shorea assamica* in Malaysia and *Pinus lambertiana* in the USA (Mittal *et al.* 1990) whereas *C. clavatum* has been reported on seeds of *Eucalyptus tereticornis* in India (Mohanan and Sharma

1991). Ivory (1987) and Domsch *et al.* (1980) illustrated the distinctive conidiophores and spores of *Cylindrocladium*. These spores suggest that it may be possible to identify infested seed lots using the seed washing technique described earlier.

The fungus is fairly easily isolated by plating surface-sterilized seed or host tissue on to standard culture media such as PDA. (Phipps, pers. comm.) recommends home-made PDA amended with 100 ppm (100 mg l^{-1}) each of chloramphenicol and chlortetracycline for isolating *Cylindrocladium* from plant tissues. The latter two components should be added only after the medium has been autoclaved and allowed to cool to 50°C. The Plant Pathologist's Pocketbook (Anonymous 1968) and Hawksworth *et al.* (1995) give procedures for making PDA. Griffin (1977) gives a selective medium for isolating *C. crotalariae* from soil which should also work for other species of *Cylindrocladium* on tree seeds.

The ingredients and procedures for making this medium called Sucrose-QT medium, are:

Sucrose (to give an osmotic potential of –1000 kPa)...............70 g
DL-Tyrosine...400 mg
KH_2PO_4...1 g
$MgSO_4 \cdot 7H_2O$...500 mg
Streptomycin sulphate...50 mg
Chlortetracycline HCl...50 mg
Bovine ox gall powder...4 g
Pentachloronitrobenzene (PCNB)..75 mg[+]
Thiabendazole..2.3 mg[+]
Dimethyldicoco ammonium chloride
(1 ml Adogen 46 [Ashland Chemical Co.])..........................750 mg
Methyldodecylbenzyltrimethyl ammonium chloride.......400 mg
Methydodecylxylenbis (trimethyl ammonium chloride), added as 1 ml of Hyamine 2389, a mixture of two quaternary ammonium compounds (Rohm and Haas)........................ 100 mg
Agar..20 g
Distilled water..1000 ml
The medium is adjusted to pH 4 before autoclaving.

Newhouse and Hunter (1983) tried two media for isolating species of *Cylindrocladium* and *Fusarium* from roots and stems of tree seedlings. Both were equally effective for isolating *Cylindrocladium* spp. The recipe for the least complicated and more effective of the two for isolating *Fusarium* spp. follows:

Glucose–Yeast Extract Rose Bengal Agar (GYRA):
Glucose..100 g
Yeast extract (Difco)..5 g
Agar...15 g

Rose bengal ....0.5 g
Distilled water ....1000 ml
Mix and autoclave these ingredients, allow the medium to cool to
60–70°C then add:

Aureomycin..50 mg
Chloramphenicol ....50 mg

Plate surface-sterilized seeds onto either of the above media
and incubate the plates at 20–25°C for 5–10 days. Use a
stereomicroscope to detect *Cylindrocladium* spp. growing from
infected seeds. Confirm identification by examining cultures for
the characteristic spores and conidiophores of *Cylindrocladium*
(Domsch *et al.* 1980; Ivory 1987). Peerally (1991), Schubert *et al.*
(1989) and most recently Crous and Wingfield (1994) give
taxonomic keys for identifying species of *Cylindrocladium*. Crous *et
al.* (1992) also describe how cultural conditions affect vesicle and
conidiospore morphology of species of *Cylindrocladium* and the
closely related genus *Cylindrocladiella*.

7.7   Isolating *Botryosphaeria* spp.

Species of *Botryosphaeria* (Pleosporales, Botryosphaeriaceae) have
conidial states in six form genera, including *Lasiodiplodia* and
Sphaeropsis (Sinclair *et al.* 1993). *Lasiodiplodia theobromae* (=*Diplodia
gossypina*) and *Sphaeropsis sapinea* (=*Diplodia pinea*) are two closely
related pathogens that cause severe losses of pine seeds in a variety
of widely geographically separated areas. For example, *Lasiodiplodia
theobromae*, a widespread, unspecialized rot pathogen with a wide
host range, causes seed rot in slash pine seeds (*Pinus elliottii* var.
elliottii) in the southeastern USA (e.g. Fraedrich 1996) and rotting of
cones and seeds of this species in South Africa (Cilliers *et al.* 1995)
and seed rot of *P. caribaea* and *P. oocarpa* seeds in Central America
(Rees 1988; Rees and Webber 1988). *Sphaeropsis sapinea*, besides
causing seed rot in slash pine seeds (Fraedrich 1996), is a foliage
pathogen of numerous pine species in over 25 countries in both
hemispheres (Swart and Wingfield 1991). Undoubtedly it attacks
seeds of many of these pines. Cilliers *et al.* (1995) developed the
following selective medium to isolate *L. theobromae* from surface-
sterilized (3.5% (m/v) NaOCl for 5 min) *P. elliottii* seeds:

Malt agar (malt extract agar) ....33.6 g
Distilled wate..1000 ml
Autoclave this basal medium for 20 min, then add (per millilitre of
the basal medium)

Tannic acid...3 g [/ml]
Benodanil ....50 mg[†]
Tridemorph ....0.5 mg[†]

Etaconazole...0.1 mg[+]
Chlorothalonil ....5 mg[+]
Rose bengal...50 mg
Use distilled water to make the stock solutions or suspensions.

Incubate the Petri plates at 25°C for 4 days under near-ultraviolet (black) light to induce sporulation of *L. theobromae*.

Swart *et al.* (1987) give a selective medium for isolating *S. sapinea* from *Pinus radiata* needle and stem tissue which is also effective for isolating the fungus from seeds. We suggest that seeds be surface-sterilized, e.g. as for isolating *L. theobromae,* before they are plated onto the following medium:

Malt extract...20 g
Agar...20 g
Distilled, deionized water....................................1000 ml
Autoclave this basal medium for 15 min, allow it to cool to 50°C, then add the following fungicides (per millilitre of the basal medium):

Rose bengal...50 mg
Benodanil ....10 mg[+]
Chlorothalonil ....1 mg[+]
*O*-phenylphenol ....1 mg
Agitate the medium for 2 min after adding the components mentioned above, and incubate the inoculated plates at 25°C.

7.8 Isolating *Colletotrichum* species

Fungi in the genus *Colletotrichum*, such as *C. gloeosporioides* (conidial state of *Glomerella cingulata*, Phyllachorales, Melogrammataceae) often occur on seeds of trees (Fig. 18) and woody shrubs. For example, a *Colletotrichum* sp. was isolated from *Eucalyptus citriodora* seeds in India (Mohanan and Sharma 1991) and *C. gloeosporioides* from seeds of the woody shrub Lupinus arboreus in New Zealand (Dick 1994). *C. dematium* has been found on seeds of *Dipterocarpus alatus*; *C. gloeosporioides* on seeds of *Dalbergia cochinchinensis*, *D. cultrata* and *Pterocarpus macrocarpus*; and another *Colletotrichium species* on *D. alatus* and *Shorea siamensis* seeds in Thailand (Pongpanich 1990). Mittal *et al.* (1990) report these and other species of *Colletotrichum* on seeds of seven tropical tree species in Malaysia, the Philippines and India plus *Acer palmatum* in South Korea.

Species of *Colletotrichum* are ordinarily isolated from surface-sterilized tissues plated onto media such as PDA. However, if seeds or fruits contain an inordinate amount of debris or soil-inhabiting microbes, other techniques may prove useful. For example, Russo *et al.* (1983) isolated *C. gloeosporioides* from Indian
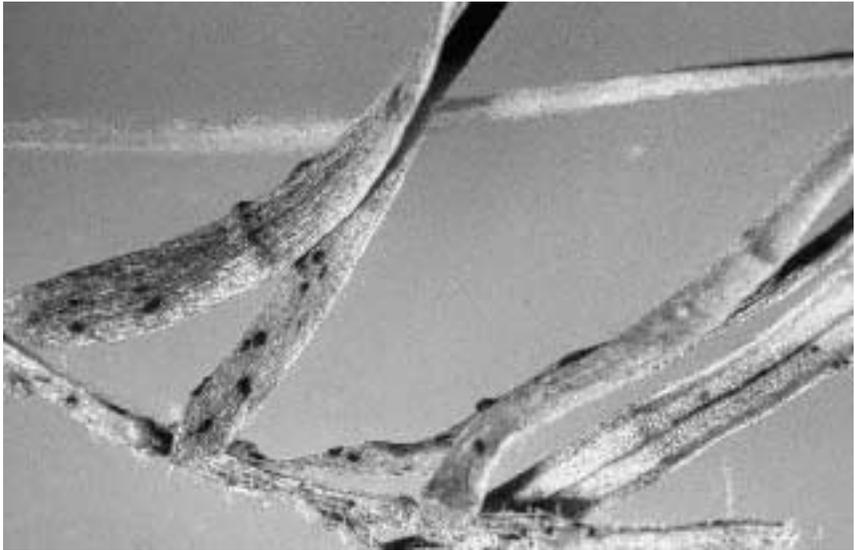
Fig. 18. Fruit bodies of *Colletotrichum acutatum* on western hemlock needles. (Dr J. Sutherland, Victoria, BC, Canada)

coral tree (*Erythrina variegata* var. *orientalis*) leaf tissues washed in warm, soapy water, rinsed in deionized, distilled water, plated onto PDA (Difco) and incubated at 22–24°C in the dark. Eastburn and Gubler (1992), studying *C. acutatum* survival in soil, isolated the fungus from strawberry (Fragaria spp.) petiole pieces by washing them in running tap water for 10–15 min, followed by a 30 s soak in 0.5% NaOCl and then a rinse in sterile, distilled water. The pieces were then plated onto acidified PDA or PDA containing benomyl (5 mg a.i. l^{-1}), streptomycin and tetracycline (each at 30 mg l^{-1}) followed by incubation at 24°C for 3–4 days.

Sutton (1992) gives the cultural and spore characteristics and many hosts for 39 species of *Colletotrichum*, including *C. gloeosporioides* and *C. acutatum* and *C. acutatum* f. sp. *pinea* which are among the most common species found on tree seeds and fruits.

7.9　Isolating *Trichoderma* species

Trichoderma species (Deuteromycotina, Hypocreaceae) are among the most common fungi occurring on seeds and fruits. It is rare to find seedlots in which some seeds do not harbour these fungi. They are so common that sometimes they may not be recorded, but disregarded as contaminants. Mittal *et al.* (1990) give 30 records of *Trichoderma* species on seeds of various hardwoods and conifers around the world. The role of *Trichoderma* spp. on seeds is not well understood, as these species may be beneficial antagonists to plant-pathogenic

fungi, or they may indicate poor quality seeds. Conversely, some high-quality Douglas-fir seedlots may harbour large populations of *Trichoderma* spp. (Bloomberg 1996). Sometimes, as with *Abies* spp. seeds (Edwards and Sutherland 1979), removing Trichoderma with surface sterilants does not improve seed germination.

The ingredients and procedures to make the *Trichoderma* selective medium of Papavizas and Lumsden (1982) are:

Glucose..1 g
Agar...20 g
Sodium propionate ....500 mg
V-8 juice ....200 ml
Distilled water ....800 ml

Combine these ingredients, autoclave, allow the medium to cool to 50°C, then add the following:

Neomycin sulphate (1%)..100 mg
Bacitracin..100 mg
Penicillin G...100 mg
Chloroneb hydrochloride ....25 mg
Nystatin...20 mg
Alkylaryl polyether alcohol..2 ml

Thoroughly mix these ingredients into the medium, e.g. using a magnetic stirrer, then disperse the medium into Petri dishes. Plate seeds or fruits, or parts thereof, on to this medium and incubate at 20–25°C, preferably using ambient lighting to stimulate *Trichoderma* sporulation. Check the plates 2–4 days later for *Trichoderma* growth which at least for the common *T. viride* is characteristically green-blue (Fig. 19).
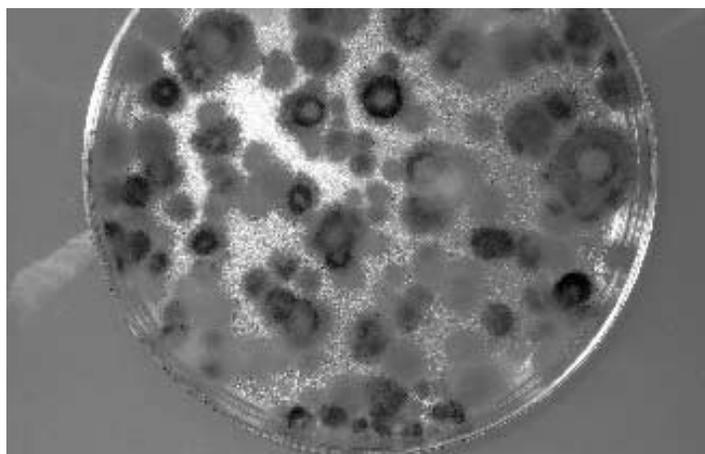
Fig. 19. Blue-green colonies of *Trichoderma* sp. following isolation from Douglas-fir seeds. (Dr J. Sutherland, Victoria, BC, Canada)

There are several taxonomic keys to certain species of *Trichoderma*, e.g. Domsch *et al.* (1980) give detailed descriptions and a key to six soil-borne species while Bissett (1984) and Rifai (1969) describe and give keys for six and nine species, respectively.

7.10 Isolating *Botrytis* species

The genus *Botrytis* (Deuteromycotina, Hyphomycetes), anamorph of the genus *Botryotinia*, contains some of the most ubiquitous fungi in the world. They are both pathogenic and saprophytic on a wide variety of plant material and tissues. Grey mould, caused by *B. cinerea*, is one of the most damaging forest nursery diseases. Mittal *et al.* (1990) recorded this fungus on the seeds of 15 tree species in at least a dozen countries, e.g. seeds of *Acer* spp. in South Korea, *A. saccharum* and *Betula papyrifera* in the USA, *Eucalyptus* spp. and *Cassia fistula* in India, *Larix decidua* in the former USSR, *Pinus caribaea* in Cuba and *Quercus* spp. in the former Czechoslovakia. Yuan *et al.* (1990) showed that *B. cinerea*, isolated from tree seeds, significantly reduces emergence of *Acacia auriculiformis* and *Casuarina cunninghamiana*, but not *Eucalyptus camaldulensis* seeds.

Botrytis spp. are readily isolated from surface-sterilized tissue plated onto PDA and other culture media. Another diagnostic tool is to incubate non-sterilized seeds or tissues in a humidity chamber at room temperature where *Botrytis* quickly fruits on affected tissues. *Botrytis* spp. are easily detected during routine seed germination testing (e.g. Prochazkova and Jancarek 1990) and the blotter technique, both of which we describe earlier. Since all *Botrytis* species form sclerotia (Jarvis 1977) these could be detected by examining seed washings (described earlier).

Fig. 20. Distinct conidiophores, and spores, of *Botrytis cinerea* (Ms R. Sturrock, Pacific Forestry Centre, Canadian Forest Service, Victoria, BC, Canada)

Botrytis spp. are readily isolated from seeds and other plant materials, and usually sporulate readily (Fig. 20), so there has not been much need for selective media for their isolation. As Jarvis (1977) points out those selective media which have been developed have been for distinguishing various species of *Botrytis* (e.g. Netzer and Dishon 1967), rather than for isolating *Botrytis* species. However, Kerssies (1990) gives a selective medium for isolating *B. cinerea* from spore traps. It could be useful for isolating this fungus from tree seeds and fruits, particularly where contaminating fungi, especially air-borne spores of *Penicillium*, *Aspergillus* and other fungi and bacteria as well, are of concern. The ingredients and procedures for making this medium follow:

Glucose..20 g
NaNO$_3$..1 g
K$_2$HPO$_4$ ....1.2 g
MgSo$_4$·7H$_2$O..200 mg
KCl ....150 mg
Agar...25 g
Distilled water ....1000 ml

Autoclave for 20 min, allow to cool to 65°C, then add:

CuSO$_4$..2.2 g
Tannic acid..5 g
PCNB ....15 mg[+]
Maneb ....10 mg[+]
Chloramphenicol ....50 mg
Fenarimol ....0.1 ml[+]

Adjust the pH of the medium to 4.5 with 5.0 N NaOH before dispensing it to Petri dishes. Plate either surface-sterilized or unsterilized seeds, or fruits or pieces of cones, onto the medium and incubate at room temperature. Most *Botrytis* species grow fast, thus regardless of the isolation technique used, observe the cultures daily for up to 7 days. To induce sporulation Jarvis (1977) recommends growing cultures of *Botrytis* spp. at 21°C in Pyrex glass containers and exposing them to near-ultraviolet light (wavelength 320–380 nm). The characteristic conidiophores and spores are easily distinguishable, and often dark sclerotia form in culture. Hennebert (1973) gives a taxonomic key to species of *Botrytis* and *Botrytis*-like genera and Jarvis (1977) lists some 22 *Botrytis* species, plus another three possibly valid species.

7.11 Isolating saprophytic or weakly pathogenic fungi

Fungi such as those in the genera *Penicillium* and *Aspergillus* are ubiquitous on tree seeds, fruits and cones. For example, Mittal *et*

al. (1990) list almost 200 records of *Aspergillus* species on numerous species of tree seeds. While such fungi are sometimes weakly pathogenic (e.g. Mittal and Wang 1986; 1987) their presence often indicates that the seeds have been previously weakened by factors such as harsh handling or improper extraction procedures, or that the seeds are immature (Bloomberg 1966). Furthermore, the presence of xerotolerant species of both *Aspergillus* and *Penicillium* can have devastating effects on the vigour and viability of seeds that are stored under RH conditions that permit their proliferation. Such conditions are common in the tropics and sub-tropics.

Usually *Penicillium* and similar fungi are isolated without difficulty by surface-sterilizing and plating seeds onto PDA or malt extract agar media. These fungi may also be detected using either the routine seed germination assay or the blotter paper technique described earlier. Since they seldom grow well on media which are selective for other fungi they can sometimes be detected by plating seeds onto such media where their growth is confined to the seeds. Singh *et al.* (1991) give the recipe for a medium (DG-18) for isolating species of *Aspergillus*, *Penicillium* and *Fusarium* plus recipes for purifying isolates of these fungi. The ingredients and procedures for making the DG-18 medium are:

Glucose...10 g
Peptone ....5 g
KH_2PO_4 ....1 g
$MgSO_4 \cdot 7H_2O$...500 mg
Dicloran..2 mg
Chloramphenicol ....100 mg
Glycerol...220 g
Agar...15 g
Distilled water ....1000 ml

Dissolve the ingredients in the water, then add the glycerol just before autoclaving. Plate the surface-sterilized seeds onto the medium in Petri plates and incubate at 25°C for 7 days under 12-h, alternating cycles of near ultraviolet (NUV) light and darkness to induce fungus sporulation. For NUV use black light tubes, e.g. Philips TLD 36 W/08, suspended about 40 cm above the seeds (20 cm between the tubes). If these tubes are not available, use cool, white florescent light tubes, such as Philips TLD 36 W/84, which emit some NUV light. Examine the fungi using either a stereo- or compound microscope.

Singh *et al.* (1991) give the cultural and spore characteristics for many species of *Penicillium*, *Aspergillus* and *Fusarium*. Domsch *et al.* (1980) give taxonomic keys for numerous species of *Penicillium* and *Aspergillus*.

8 Molecular approaches to fungus detection

Molecular biological and biochemical approaches to the detection, identification and quantification of micro-organisms *in situ* carry certain advantages over, and are complementary to, traditional culture-based methods of analysis. In addition to helping avoid laborious and time-consuming aspects of laboratory culture, such methods circumvent the inherent bias in favour of organisms that are most amiable to culture on commonly used laboratory media. In fact, it has long been recognized that some important groups of micro-organisms may be completely resistant to laboratory culture (Skinner *et al.* 1952). The following is necessarily brief and is intended as a starting-point for the interested reader.

Culture-independent methods target microbial nucleic acid (DNA or RNA), protein or lipid molecules that can be distinguished from that of the host (in this case, plant), and such informative molecules are collectively referred to as biomarkers (White *et al.* 1999). Methods for detection and quantification of these biomarkers generally arrive in fields such as plant pathology and microbial ecology as a function of 'trickle-down' from clinical biology. Therefore, despite their growing popularity throughout the biological sciences, it is the medical literature that must be surveyed in order to appreciate fully the potential impact that these approaches hold in monitoring microbial populations growing on non-orthodox seeds or embryonic axes during, and prior to, storage.

The most recent non-clinical review addressing the molecular detection of fungi was provided by Kowalchuk (1999). Microbial DNA and RNA are usually detected by use methods based upon the polymerase chain reaction (PCR), which is used to amplify nucleic acid fragments specific to the desired taxonomic level, which may range from microbial strain to one of the three domains of life, or target groups of viruses. In studies related to microbial infection of plant material, such methods must be able to discriminate between the target populations and the host genetic material. Furthermore, the genetic marker chosen must contain the necessary information to identify the microbial population(s) of interest. Amplification products may be subjected to a number of secondary analyses, designed to glean information on the presence and identity of target populations. Primary purposes are detection of a specific microbial species or strains (presence/absence and

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[†]   Contributed by John R. Stephen and Bill Finch-Savage, Crop and Weed Science Dept, Horticultural Research International, UK and George A. Kowalchuk, Dept Plant–Micro-organism Interactions, Netherlands Institute for Ecology, Netherlands.

quantification), taxonomic profiling of the target community to determine diversity and associations, and comparison of samples to detect effects of, and recovery from, perturbation. Often, a given approach provides information on more than one of the above.

8.1 Detection of pathogens by PCR

The simplest application of PCR-technology is in the detection of the presence of a particular pathogen or taxonomically related group of pathogenic species. For these purposes, all that is required is a method for extracting nucleic acids from seed tissue (numerous proprietary kits are marketed by Ambion, Biorad, Hybaid, Quaigen and others), suitable primers and reagents, and a thermocycling block. However, in this form the information recovered is not quantitative, the recovery of a PCR product warns of the presence of a pathogen, but does not indicate the severity of the infection (Henson and French 1993; Elliot *et al.* 1993; Schubert *et al.* 1999). Several methods exist to generate quantitative information from the PCR, which include spiking the reaction with a known amount of an internal standard (which may be a modified version of the target sequence; e.g. Nicholson *et al.* 1998), extinction–dilution of the template (Chandler 1998), and 'real-time' PCR, requiring expensive and specialized equipment (Loeffler *et al.* 2000).

The above methods can provide results in less than a single working day, and have considerable application in determining seed quality prior to storage. Further information on the identity of contaminant species may be derived by hybridising the PCR amplification product to taxonomically informative probes (Posteraro *et al.* 2000), or by generating a profile of the target population by denaturing gradient gel electrophoresis (DGGE, Muyzer *et al.* 1993; Kowalchuk *et al.* 1997) or ribosomal intergenic spacer analysis (RISA; García-Martínez *et al.* 1999). Both methods may be used to monitor microbial biocontrol agents (e.g. Stephen *et al.* 1999), and the former of these, DGGE (and its relatives, TTGE and TGGE) can be made to hold a quantitative aspect by use of real-time PCR or internal standards (Felske *et al.* 1998; Brüggemann *et al.* 2000). These methods target any user-definable taxonomic group, from kingdom to species, and are usually used to discover novel organisms that are resistant to laboratory cultivation: such organisms are often of more environmental relevance than their cultivated relatives (Watanabe and Baker 2000).

For simplicity, the methods described above have assumed that the user is targeting microbial DNA: however, this may not always be useful. For example, if the investigator wished to use a

molecular approach to test the efficacy of an antifungal treatment, PCR methods targeting DNA may be quite ineffectual, as DNA can be rather a stable molecule which may persist outside the cell or in dead cells for considerable lengths of time. RNA, and in particular mRNA, is a much more labile molecule, as is rapidly degraded by cellular ribonucleases following cell death. Intact RNA, free of DNA, can be readily purified from plant material (using proprietary kits, as above), and the target sequences converted to DNA prior to PCR amplification (termed reverse-transcription PCR, RT-PCR). Using such an approach, only viable, metabolically active fungi and bacteria are detected (e.g. Okeke *et al.* 2000).

In addition to nucleic acid markers, biochemical markers such as phospholipid ester-linked fatty acids (PFLAs), neutral lipid fatty acid (NFLAs), and ergosterol (Ollson *et al.* 1998), may be useful for tracking some microbial groups. Such techniques avoid biases that may be introduced by the amplification of nucleic acid biomarkers, but they usually offer only a coarse level of resolution.

Given the rapid advance in the variety and validity of molecular biological approaches for the detection and characterization of microbial populations, it is likely that these methods will help improve the selection of the highest quality non-orthodox seed material for cryopreservation and monitoring contamination of seed during prolonged storage.

9 The nature of viruses on forest tree seeds[§]

Viruses can be widely disseminated in tree seeds. Thus, they threaten not only those hosts on which they are seed-borne, but, via secondary spread, other hosts as well. Viruses can be carried either on the seedcoat (and mechanically infect germinants during seed germination) or within the seed embryo as the result of infection via the ovary or from pollen of infected mother plants. Transmission occurs either by way of the sperm cell nucleus or cytoplasm, or from virus particles on pollen. All pollen-transmitted viruses are also seed-borne. The International Committee on Taxonomy of Viruses recognizes 32 groups of well-characterized viruses, 12 of which commonly occur in trees and shrubs (Cooper *et al.* 1986). Nepoviruses, ilarviruses and cucumoviruses are transmitted via tree seeds or pollen. Holliday (1990) gives sizes, physical and chemical characteristics and related information on these groups of viruses

Nepoviruses, common in trees and shrubs, are transmitted by seed and pollen, or by nematodes or other means. *Cherry leaf roll virus* (CLRV), for example, is prevalent in species of *Betula* and *Cornus*, and also in *Fagus sylvatica*, *Fraxinus excelsior*, *Juglans regia*, *Rhamnus cathartica* and *Sambucus nigra*, where symptoms include chlorotic ringspots on leaves, leaf dwarfing and eventually tree decline. Another nepovirus, *Arabis mosaic virus*, occurs in ash (*Fraxinus* spp.). Ilarviruses tend to be host specific; they are widespread and cause serious losses in fruit trees. *Elm mottle virus* affects *Syringa vulgaris* and *Ulmus glabra*, whereas *Apple mosaic virus* occurs in *Rosa* spp. and *Corylus avellana*, and *Prunus necrotic* ringspot virus is found in *Aesculus hippocastanum*. *Cucumber mosaic virus*, a cucumovirus, has been detected in *Lonicera periclymenum* and *Ligastrum* species. Tobamoviruses occur on seed coats and infect germinants, e.g. of *Quercus robur* and *Acer* spp. during seed germination. However, they are not internally seed-borne (Büttner and Führling 1996; 1997).

Three different techniques are presented here for detecting viruses in hardwood seeds or young plants, the seeds of which are suspected of harbouring viruses. Because of the irregular distribution and relatively low concentration of viruses in seed lots, these protocols are more reliable than techniques such as transmission electron microscopy to examine seed or plant extracts for viruses. However, serological techniques, such as ELISA, may

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suffer from problems, for instance the presence of phenolic compounds in seeds and other tissues. Our protocols can be used for either seeds or leaf tissue of germinants. In the case of seeds, the seed coat may be removed when preparing samples for assay.

The first technique is the double antibody sandwich ELISA (enzyme linked immunosorbent assay), which is recommended for detecting ilarviruses and cucumoviruses. Cooper *et al.* (1986) used this extremely sensitive immunological technique to detect ilarviruses in cherry (*Prunus avium*) seed, and to survey poplar germplasm for Poplar mosaic virus. There are no reports on ELISA being used to detect cucumoviruses in forest tree seeds, but studies on herb and other seeds confirm its reliability.

The second technique is the immunocapture–reverse transcriptase polymerase chain reaction (IC-RT-PCR), which is used for detecting the CLRV nepovirus. Our experience suggests that CLRV is one of the most common seed-and pollen-transmitted viruses affecting hardwood trees and shrubs. Recently Büttner *et al.* (1996) used grafting to transmit the pathogen to host plants, and a modified polymerase chain reaction (PCR) technique, and the hybridization technique to detect CLRV. The PCR and hybridization techniques detect small amounts of viruses. Werner *et al.* (1997) evaluated a method for detecting CLRV in seeds of birch and concluded that PCR followed by immunocapture–reverse transcriptase is the most sensitive method for detecting viral RNAs. Cooper (1993) showed that the seed transmission rate of CLRV is very variable and depends on whether male or female gametophytes originated from a CLRV infected tree. The rate of vertical spread of viruses within the host depends on various factors such as virus strain and environmental conditions (Maule and Wang 1996).

The third technique is the dot blot hybridization, which is used for detecting tobamoviruses in oaks and maples. It can be used to detect specific plant RNA viruses or viroids when a specific probe is available. This probe must be produced by molecular biological methods. In comparison to IC-RT-PCR, it is not absolutely necessary to have information concerning the genome sequence to construct a probe. The dot blot hybridization technique is particularly useful for routine testing of many samples. PCR, which is more specific than the hybridization technique, is useful for differentiating virus strains, but this as well as the equipment and chemicals are expensive and so this technique is not recommended for routine virus diagnosis.

10   Protocols for detecting viruses

10.1 Double antibody sandwich, enzyme-linked immunosorbent assay (DAS-ELISA)

As given by Clark and Adams (1977) and van Regenmortel (1982). Note: If bacterial contamination is prevalent, add 0.02% sodium azide to all buffers.

Recommended for detecting ilarviruses and cucumoviruses.

10.1.1 Antibody preparation

Prepare the globulins by precipitation from the antiserum with an equal volume of 4 M ammonium sulphate. Slowly stir the solution for an h at room temperature, then centrifuge it at 8000 rpm for 10 min. Re-dissolve the pellet in 1 ml of half concentration, phosphate-buffered saline (PBS), pH 7.4, then remove the ammonium sulphate by dialysis using half concentration PBS.

10.1.2 Coating to adsorb antibodies to the plate

Antibodies are diluted in coating buffer, usually 1:1000 (but the appropriate dilution should be determined when initiating the procedure, as the rate depends on the quality of the antibodies). Use 200 µl/well for coating by passive adsorption, incubate at 37°C for 4 h, wash three times with PBS-T, i.e. PBS containing 0.05% Tween 20, with 3 min intervals between washes.

10.1.3 Adding the virus

Extract the viral antigens in seeds or leaf material by using 1:10–1:30 in PBS-T. Then transfer 200 µl of the sample into the wells, incubate overnight at 4°C, then wash three times with PBS-T, using 3 min intervals between washes. To reduce non-specific reactions and increase the sensitivity of virus, add 1–2% polyvinylpyrrolidone, 1 M urea and reducing agents to the buffer (Guggerli,1979).

10.1.4 Blocking to improve the results (optional)

Block with 200 µl 1% bovine serum albumin (BSA) per well and incubate for 2 h at room temperature. Then wash three times with PBS-T with 3 min between washes.

10.1.5 Adding enzyme-labelled virus antibody (conjugation)

Dilute (usually 1:500–1:1000, but determine dilution empirically) antivirus enzyme conjugate in PBS-T. Next transfer 200 µl/well and incubate at 37°C for 4 h, then wash three times with PBS-T, using 3 min intervals between washes. The most common enzyme

conjugate is prepared with alkaline phosphatase (Boehringer, Mannheim or Sigma, St. Louis, Missouri, USA) by coupling the globulins with enzyme at 1 ml : 0.1 ml (v/v globulin : enzyme) using 0.06% glutaraldehyde. Store the conjugate at 4°C in the presence of 1% bovine serum albumin.

10.1.6   Adding the substrate

Dilute, just before using, the substrate *p*-nitrophenyl phosphate (1 mg ml^{-1}) in 0.1 M diethanolamine buffer, pH 9.8. Transfer 250 µl into each well. Score the results visually by intensity of the yellow colour, or by either reading the absorbance at 405 nm using a spectrophotometer, or an ELISA-reader. Add 50 µl of 3 M NaOH to each well to stop the reaction, when the background color of the blank/healthy controls begin to darken, or after the positive colorimetric reactions are recorded. The results are positive if the absorbance is twice that for healthy material (controls), or two standard deviations higher than the mean of a negative control curve.

10.2 Immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR)

See Büttner *et al.* (1996) and Werner *et al.* (1997).
   Recommended for detecting the nepovirus CLRV.

10.2.1   Sample preparation

Grind the plant tissue under liquid nitrogen, then homogenize the powder in 1:10 (w/v) buffer (PBS–Tween, 2% (w/v) poly-vinylpyrrolidone 40 (PVP 40). Centrifuge plant sap for 10 min at 3000*g*.

10.2.2   Coating and reverse transcription

Coat sterile, 0.5 ml, reaction tubes (Eppendorf Safelock) with 50 µl purified CLRV specific antibodies (40 µg/ml in 50 mM carbonate buffer, pH 9.6). Then incubate for 3 h at 37°C. Next wash the tubes three times for 3 min with 150 µl PBS–Tween (20 mM phosphate buffer, pH 7.4, 135 mM NaCl, 0.05% (v/v) Tween 20). Incubate the coated tubes with 50 µl of the plant extracts (see sample preparation) overnight at 4°C. Afterwards wash three times for 3 min each with 150 µl PBS–Tween, then centrifuge the tubes briefly and remove the remaining wash buffer.

   Then add buffer and the specified components to a total volume of 20 µl: 50 mM Tris–HCl, pH 8.3, containing 75 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, 1 mM dNTPs , 20 U ribonuclease inhibitor (Promega, Madison, Wisconsin, USA), 200 U M-MLV reverse transcriptase (GIBCO BRL, Inchinnan), 100 pmol CLRV specific

first strand primer (5´-GTC GGA AAG ATT ACG TAA AAG G-3´). Incubate the captured virions for 1 h at 37°C, denature at 95°C for 3 min, then amplify aliquots of 2 µl of the reverse transcription reaction in a subsequent PCR.

10.2.3   Polymerase chain reaction

Mix 2 µl of the reverse transcription reaction, 20 pmol first strand primer, 20 pmol second strand primer and 2.5 U *Taq*-DNA-polymerase. Then add 100 ml buffer (10 mM Tris–HCl, pH 8.3, containing 1.5 mM $MgCl_2$ and 50 mM KCl). Next, amplify the viral cDNA in a thermocycler at 35 cycles, anneal the primer at 51°C for 60 s, elongate the chain at 72°C for a 60 s, then denature at 95°C for 30 s.

10.2.4   Gel electrophoresis of the amplification products

Melt 2 g agarose in 100 ml 1 × TBE-buffer (10 × TBE: 0.9 M Tris, 0.9 M boric acid, 50 mM EDTA), cool to it to about 60°C, then add 1 mg of ethidium bromide/ml of solution). Now transfer the agarose into a electrophoresis chamber (e.g. Biometra midi-gel), then prepare samples (aliquots of the PCR products) by adding 1:1 loading buffer (8 ml of 87% glycerol, 4 ml 10 (TBE, 28 ml distilled water, 0.01% bromephenol blue, 0.01% xylencyanol). Lastly, do the electrophoretic separation at 70 mA and observe the nucleic acid bands under UV-light.

10.3 Dot blot hybridization

(Miltenburg *et al.* 1995). Use sterile Eppendorf tubes and DEPC-treated distilled water to make the buffers.

   Recommended for detecting tobamoviruses.

10.3.1   Extracting RNA from plant tissue

Grind 200 mg plant tissue in a mortar with liquid nitrogen, to a fine homogenous powder, transfer it to a sterile Eppendorf tube, then add 800 µl denaturation buffer (4 M guanidinium isothiocyanate, 0.2 M mercaptoethanol) and vortex the mixture. Next, centrifuge it at 12000 rpm for 5 min, transfer 500 µl of supernatant to a fresh tube. For the chloroform–phenol extraction add 250 µl phenol and 250 µl chloroform/isoamylalcohol (24:1 v/v). Then vortex the mixture, centrifuge it at 12000 rpm for 3 min, repeat the chloroform/phenol extraction twice. Next transfer the combined supernatants to a fresh tube, add 500 µl chloroform, centrifuge at 12000 rpm for 3 min, vacuum off the aqueous phase and transfer to a fresh tube.

   Ethanol precipitation may be used for leaf or soft woody tissues by adding 50 µl 4 M LiCl and 1.3 ml 96% ethanol before vortexing.

When assaying seeds, Rosaceae leaf tissue or hard woody tissues, add 60 µl 6 M NaCl and 1.3 ml 99.6% ethanol, vortex, then store for either 1 h at –80°C, or overnight at –20°C, or for 1 min on ice. Then centrifuge at 10000 rpm for 30 min, remove and discard the supernatant, add 1 ml 70% ethanol and continue sample preparation or store at –80°C until needed.

10.3.2   Sample preparation
Centrifuge the tubes at 15000 rpm for 20 min, remove and discard the ethanol, add 500 µl 70% ethanol and vortex centrifuge at 15000 rpm for 10 min, remove and discard the ethanol, add 500 µl of 70% ethanol and vortex, centrifuge at 15000 rpm for 10 min, remove and discard the ethanol, then air dry the pellet or dry it by speed vacuuming it twice for 2 min each time.

10.3.3   Blotting procedure
Re-dissolve the pellet in 200 µl formaldehyde, 6 M in $10 \times SSC$ (1.5 M NaCl, 0.15 M trisodiumcitrate), incubate 15 min at 65°C, chill on ice, wet a positively charged nylon membrane with sterile water and incubate for 15 min in $20 \times SSC$, incubate blotting paper (Whatman 3mm) for 15 min in $20 \times SSC$. Next wash the sample wells of the blotting apparatus with 400 µl $20 \times SSC$, load RNA samples into the sample wells (determine the concentration) and incubate for 30 min at room temperature. Finally, pass the RNA samples through the membrane by applying suction, wash with 500 µl $20 \times SSC$ and incubate the membrane for 1 h at 80°C, or UV-crosslink the membrane ($2000 \, \mu J \times 10^3$).

10.3.4   Hybridization
Transfer the membrane, print side up, into a hybridization flask, add 10 ml hybridization buffer ($5 \times SSC$, 0.1% N-lauroylsarcosin, 0.02% SDS, 50% formamide, 2% blocking reagent (Boehringer, Mannheim) and 125 µg/ml herring sperm DNA) and incubate it for 1 h at 68°C with continuous agitation. Then add 50 µl of hybridization probe (Büttner and Führling 1997), incubate overnight at 68°C, remove the hybridization buffer (which can be reused several times), wash the membrane three times (15 min each) in 30 ml of wash solution I ($2 \times SSC$, 0.1% SDS) at 65°C, then wash the membrane twice for 5 min each in wash solution II ($0.1 \times SSC$, 0.1% SDS) at room temperature.

10.3.5   Detection
Equilibrate the membrane in buffer I (0.1 M maleic acid, 0. 15 M NaCl with 0.3% Tween 20, pH 7.5) then block the membrane by

gently agitating it in buffer II (1% blocking reagent dissolved in buffer I) for 60 min. Next dilute Anti-DIG-alkaline phosphatase 1:10000 (75 mU ml^{-1}) in buffer II and mix gently by inversion (the solution is stable for several days at 4°C), incubate the membrane in the antibody solution for 30 min, shaking the tray gently to ensure the membrane is always covered. Afterward discard the antibody solution and wash the membrane twice for 15 min in buffer I, equilibrate the membrane in buffer III (0.1 M Tris–HCl, pH 9.5; add 0.1 M NaCl, 0.05 M MgCl$_2$) for 2 min, dilute CSPD (Cold Shock Protein D) in buffer III (1:100), place the wet membrane between two sheets of plastic page protectors. Then lift the top sheet and add diluted CSPD (0.5 ml/100 cm^2), distribute the drops over the surface by rocking the membrane; create a closed liquid seal by wiping the top sheet to remove bubbles, then incubate the membrane for 5 min at room temperature. Next seal the semi-dry membrane in a plastic bag, incubate it for 15 min at 37°C, expose the membrane to Hyperfilm-ECL (Amersham) for 15 min, then develop the film and expose the membrane again as long as needed.

11   Growing-on tests

Recommended for detecting pathogens with a long latent period between infection and appearance of disease symptoms.

Growing-on tests are especially useful for detecting seed-borne pathogens, such as viruses, which may have a long incubation period before symptom development. Consequently, growing on tests are often used by plant quarantine officials to detect pathogens which might otherwise go undetected by short-term tests or assays of seeds. The duration of such tests varies with the phenology of the host plants and the suspected pathogens. For example, seed-borne *Sirococcus conigenus* on spruce seedlings would be expected to appear when the plants are 6 weeks to 6 months old, while some virus pathogens may not appear for two years or longer in the growing plants. A representative sample of the seeds being assayed is planted in a sterile growing medium, such as a mixture of peat and sand, or sterilized soil, and the resulting seedlings are grown in a greenhouse free of the suspected pathogen. Ideally seedlings would be kept in insect-proof greenhouse to prevent insects from vectoring viruses or other pathogens. Nevertheless, the young plants may have to be sprayed with insecticides to prevent insect feeding. Plants are examined periodically for symptom development and pathogen confirmation.

Orchard (1984) used a growing on test to show the seed-borne nature of the butternut canker pathogen *Sirococcus clavigignenti-juglandacearum*. Butternuts were collected from diseased trees in the autumn and following storage at 4°C in plastic bags for 3–27 months they were sown in flats in the greenhouse. When the seedlings were 2 weeks old they were transplanted into a sterile sand–soil–peat mixture in 13 cm diameter clay pots. Three weeks after transplanting the seedlings, the pathogen was isolated from necrotic stem tissue near the point of seed attachment. Koch's Postulates were fulfilled to confirm pathogenicity of the seed-borne inoculum.

Glossary

Agar   An ingredient used to solidify culture media.

Anamorph   Asexual stage.

Autoclave   A device for sterilizing culture media, water and other materials. Most micro-organisms are killed by autoclaving (sterilising) for 15 min at 121°C, but sometimes longer times are used.

Bacterium (bacteria)   Usually one-celled (0.25–2.0 µm) micro-organisms, lacking a cellular membrane, usually without chlorophyll; parasitic or saprophytic.

Basidium (basidia)   Specialized cell or organ on which basidiospores of Basidiomycotina (basidium-producing) fungi are produced.

Blight   A disease which develops quickly, usually, affecting young tissues of foliage, fruits and the like.

Canker   A definite, dead, dry, sunken or raised area on a stem, bole of a tree and other tissues, surrounded by living tissue.

Chlorotic   Tissue with reduced chlorophyll content.

Conidiophore   A specialized fungus structure, arising from the vegetative growth, upon which conidiospores are borne.

Conidiospore   An asexual spore of a fungus.

Culture medium (media)   A substrate upon which fungi and other micro-organisms are grown.

Damping-off   A disease attacking germinants before emergence or rotting them off afterward near the ground line.

Fruiting body   A specialized fungus structure in which spores are produced.

Fungus (fungi)   A single to many-celled organism (often microscopic), lacking chlorophyll, commonly producing hyphae (thread-like vegetative growth) that together constitute a mycelium, and reproducing by spores. Most plant diseases are caused by fungi.

Haemocytometer   A slide-like device for counting micro-organisms or small particles using a microscope.

Hypha (hyphae)   The thread-like, vegetative growth of a fungus.

Inoculum spores   Vegetative growth or other structures of a micro-organism, that come into contact with plants and other organisms, or are introduced on/in a culture medium.

Koch's postulates   A set of protocols for proving pathogenicity of a micro-organism.

Malt agar   A culture medium for micro-organisms, especially fungi, in which malt extract, agar and water are the main ingredients.

Mycelium   A mass of hyphae.

Pathogen (pathogenic)   An organism, often a micro-organism, capable of causing disease. Bacteria, fungi, viruses, nematodes are examples of plant pathogens.

PDA (potato dextrose agar)   A medium commonly used for laboratory culture of fungi, the major ingredients of which are potato, dextrose (sucrose), agar and water.

Root rot   Decay of roots.

Saprophyte   An organism such a fungus or bacterium which lives on dead, organic material.

Sclerotium   A hard mass of fungus vegetative growth (hyphae) serving as a survival mechanism during unfavourable conditions.

Seed-borne   Pathogen or pest disseminated as a contaminant of seed.

Seed-transmitted   Pathogen or pest biologically and physiologically attached to the seed.

Spore   A reproductive body, one to several cells, of fungi, bacteria and certain lower plants.

Viroid   An infectious pathogen consisting of ribonucleic acid, smaller than a virus.

Virus   A submicroscopic, infectious nucleoprotein, often pathogenic, multiplies only in living cells.

Water agar   A culture medium containing only water and agar, usually 1.5–2%.

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