Germplasm health and disease indexing with particular reference to forest trees in tropical countries

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SUMMARY

This paper reviews firstly, the dissemination of phytopathogenic organisms either on, in, or together with, various forms of forest tree germplasm. It concludes that relatively few dangerous pathogens are transmitted by tree germplasm in the form of cleaned seed, pollen, or tissue cultures, but that many are disseminated by vegetative tissues, such as cuttings and scions. Secondly, it reviews traditional and modern procedures for the detection and quantification of phytopathogenic organisms which form the basis of disease indexing. Lastly, the paper contains a brief résumé of seed health testing and plant quarantine in relation to transfers of tree germplasm and concludes that rapid, cheap screening and diagnostic techniques are needed to ensure that the currently increasing transfers of germplasm are securely achieved.

Keywords: Tree germplasm health; disease indexing.

TRANSMISSION IN GERMPLASM

The major type of germplasm utilized for forest tree species is that of 'seed' (either true seed, or seed inside the fruit with or without remaining flower parts). Other types of germplasm include pollen, tissue or cell cultures, and vegetative tissues such as cuttings and scions. These can be infected with microorganisms in the following ways: (1) internally within the living cells; (2) externally in the dead outer tissues; or (3) externally on the germplasm surface. Organisms carried with associated impurities, such as pieces of cones, needles, leaves and fruits are not dealt with here as they should be excluded by modern seed cleaning procedures. Some larger pathogenic organisms, such as insects, may themselves also transmit smaller pathogenic organisms (viruses or mollicutes); this method of dissemination is also not discussed herein. This review mainly addresses the transmission of pathogenic organisms which give rise to disease in the carrier host, or other hosts. However, many other weak pathogens can destroy germplasm in storage, especially seed and pollen, particularly if storage conditions are suboptimal or the germplasm is senescent. Examples of potential transmission are given below when possible.

Transmission in tree 'seed'

Some members of all the major groups of pathogenic microorganisms and pests can be disseminated with seed (Neergard, 1969, Noble and Richardson, 1968, Richardson, 1979, Anderson, 1986) including some which cause disease of forest trees.

1. *Insects*. Tree seeds are often infected with small insects which destroy their contents (Eungwijarnpanya and Hedlin, 1984; Southgate, 1983; Sen-Sarma, Thakur and Sehgal, 1988; Singh and Rana, 1990) but few of these cause disease on other parts of growing plants. Many more pests are transported on seeds with attached parts of fruits and flowers, or with abundant extraneous matter.

2. *Nematodes*. Tree seeds are not known to transmit plant pathogenic nematodes; such transmission has, however, been reported for some cereal crops (Mathur and Lal, 1989).

3. *Fungi*. Tree seeds are often infected by large numbers of fungi (Sen-Sarma, Thakur and Sehgal, 1988; Mittal and Wang, 1987; Yuan, Old and Midgley, 1990; Singh and Rana, 1990), most of which are saprophytes living in dead tissues. However, a few of these appear to act as weak pathogens causing decay of the seed during storage, especially when held under poor storage conditions; subsequently some of these organisms may cause damping-off in the young germlings produced (Ivory, 1987). A few fungi are also known to invade the internal living tissues of tree seed and subsequently to give rise to disease of the tree after germination of the seed; examples are the systemic *Fusarium* wilt of Mimosa (Gill, 1968) and Chestnut Blight (Lanier, Joly, Bondoux and Bellemere, 1976). Some non-systemic disease fungi, such as *Glomerella cingulata*, which causes leaf and pod spots on Acacias (Sinclair, Lyon and Johnson, 1987) and certain *Fusarium* species causing a pod rot on *Leucaena* spp. (Lenné, 1991) can also spread in this way. Many other fungi which give rise to dormant propagules (e.g. *Macrophomina phaseolina* and the grass pathogen *Claviceps purpurea*) or long-lived reproductive structures (e.g. *Sphaeropsis sapinea* on cone scales of Pines

and *Mycosphaerella* species on Pine needles), may also be disseminated in these forms within impure batches of seeds (Ivory, 1987) resulting in their transmission across international boundaries.

4. *Bacteria.* Tree 'seed' are frequently heavily contaminated with bacteria, which are generally found in all dead tissues and on plant surfaces. Most of these are harmless with very few plant pathogenic bacteria known to be disseminated on tree seed; examples which have been reported include *Xanthomonas campestris* pv. (pathovar) *juglandis*, which causes bacterial blight of Walnut (Lanier *et al.*, 1976; Noble and Richardson, 1968; Sinclair, Lyon and Johnson, 1987) and *Pseudomonas fluorescens*, which causes bacterial pod rot of *Leucaena leucocephala* (Lenné, 1991).

5. *Mollicutes.* Tree seeds are not known to transmit plant pathogenic mollicutes (Mycoplasma-like organisms, Spiroplasmas and Rickettsia-like organisms) (Smith *et al.*, 1988, Sinclair *et al.*, 1987), although these organisms are often systemic in other plant parts.

6. *Viruses.* Despite the frequent systemic occurrence of viruses in plants and trees, few are transmitted in tree seed (Bos, 1977). Those that are transmitted in this way are mostly from the Nepovirus and Cucumovirus groups and have been found in seeds of Ash, Birch, Elm, Ginko, Paulownia, Poplar, Privet, Walnut and fruit trees such as *Prunus* spp. (Cooper, 1979, 1988; Cooper, Massalski and Edwards, 1984; Cooper, Kelley and Massalski, 1988; Lanier *et al.*, 1976; Murant, 1989; Smith, Archer, Dunez, Lelliot and Phillips, 1988). They are mostly carried internally in live cells of the embryo and develop to infect the germinating plant systemically.

Transmission in tree pollen

A few members in each of two major groups of pathogenic microorganism (bacteria and viruses) are known to be disseminated by plant pollen. They are amongst the smallest in size and many are also transmitted by seed (Lanier *et al.*, 1976).

1. *Bacteria.* Bacterial blight of Walnut caused by *Xanthomonas campestris* pv. *juglandis* gives rise to a blight of stems, foliage and nuts. It perennates in buds and male catkins giving rise to infected pollen which can infect a healthy mother tree through the female flower and can also give rise to infected nuts (Smith *et al.*, 1988, Lanier *et al.*, 1976; Sinclair *et al.*, 1987).

2. *Viruses.* Although most viruses become systemic in infected plants, very few become transmitted to the pollen which the plant produces (Mandahar, 1990). Those which have been detected in tree pollen are mainly from the Nepovirus and Ilarvirus groups and have been detected in the pollen of Ash, Birch, Elm, Hazel, Horse Chestnut, Lilac, Walnut and agricultural trees such as *Prunus* spp. (Cooper, 1979; Cooper *et al.*, 1984; Cooper *et al.*, 1988; Mandahar and Gill, 1984; Murant, 1989; Sinclair *et al.*, 1987; Lanier *et al.*, 1976).

3. *Insects, nematodes, fungi and mollicutes.* Insects, nematodes, fungi and mollicutes have not been reported to be associated with pollen.

Transmission in graft scions and in cuttings

Each of the major groups of pathogenic microorganisms contain many members which can be transmitted in tree shoots used for graft scions or for cuttings (Lanier *et al.*, 1976). However, the larger organisms such as insects and fungi are often readily visible and can thus be easily eliminated from consignments of such materials. Reduction in the size of the samples employed by reducing shoots to buds, or even to meristems, greatly reduces the risks.

1. *Insects.* There are many insects which can infest plant shoots and all stages from egg to adult have been found. The smallest insects or youngest stages of development can be very difficult to detect as was the case of the accidental introduction of Pine Woolly Aphid into Central and East Africa (Odera, 1974).

2. *Nematodes.* Some nematodes which invade the shoots and foliage of plants can be readily transmitted in shoots used for propagation purposes. An example is that of the Pine Wood Nematode (*Bursaphelenchus xylophilus*), which affects several Pines in Japan, Taiwan, Korea and Hong Kong (Ivory, 1987) and is open to transmission in this way.

3. *Fungi.* Many fungi which invade the shoots and foliage of trees can be readily transmitted in shoots used for propagation purposes. However, most can be readily detected by visual examination and rejected, except in the case of systemic diseases such as Fusarium Wilt of Mimosa and Sissoo, etc., and of diseases which have extended symptomless incubation periods e.g. *Cyclaneusma minus* on needles of *Pinus* spp. (Ivory, 1987).

4. *Bacteria.* Phytopathogenic bacteria such as *X. campestris* pv. *juglandis* can infect all parts of Walnut host trees including the shoots and buds. Use of buds and shoots for propagation purposes can therefore readily disseminate the disease.

5. *Mollicutes.* These organisms are usually systemic throughout the aerial parts of plants and are thus likely to infect all shoots of infected trees used for propagation. Most mollicutes pathogenic to trees are known to be readily disseminated with graft scions and cuttings (Raychaudhuri and Varma, 1988; Tsai, Chen, Shen and Jin, 1988; Chiykowski, 1988).

6. *Viruses.* Most plant viruses are present systemically in their host and are thus likely to be present in all shoots used for propagation derived from infected trees. Most tree viruses are known to be readily transmitted in graft scions (Cooper, 1988; Uyemoto, 1989; Sinclair, Lyon and Johnson, 1987; Lanier *et al.*,

1976), a characteristic used in the traditional transmission bioassay procedure.

Transmission in tissue cultures

Most pests and pathogens, if present, become readily apparent in tissue culture dishes because of the favourable environmental conditions under which the cultures are grown. Tissue cultures kept for some time are therefore often regarded as pathogen free unless obviously infected. However, some microorganisms can co-exist with the host cells in tissue cultures and are therefore disseminated to all the derived cultures and to any plants produced from them. Mollicutes and viruses are not easy to observe directly and modern techniques are required for their detection.

1. *Fungi.* Fungi can contaminate tissue cultures, giving little sign of their presence (Duhem, Mercier and Boxus, 1988), especially those derived from symptomless endophytes. No examples have been found for the dissemination of tree pathogens in this manner, however.

2. *Bacteria.* Bacteria are frequent contaminants of tissue culture (Duhem, Mercier and Boxus, 1988; Scortichini, 1988) but, as for fungi, pathogenic species are not known to spread in this manner. They can be endophytic in origin.

3. *Mollicutes.* If present in the host cells from which the cultures are derived, these organisms will contaminate all the cells and tissues which are subsequently produced (Chiykowski, 1988). However, as seeds are not infected, cultures derived by careful preparation of embryos and other seed tissues will not normally contain mollicutes.

4. *Viruses.* Many viruses can co-exist with plant cells in tissue cultures which are derived from infected host tissue (Monette, 1988). Tree seeds, however, are mostly virus-free, except for those described earlier, and some cells in apical meristems may also be virus-free; these tissues are relatively safe tissues for culturing, but other tissues are frequently infected.

DISEASE INDEXING IN TREE GERMPLASM

One of the pre-requisites for any scheme to try to prevent the dissemination of diseases in germplasm transfers is to be able to detect and quantify the presence of phytopathogenic organisms in the germplasm. Methods should preferably be rapid, reliable, cheap and simple to operate if such transfers are to become commonplace. Some of the traditional and newer techniques are detailed and discussed below. Diagnostic techniques and problems for fungi, bacteria and viruses have recently been reviewed by Waller (1989), Civerolo (1989) and Stace-Smith and Martin (1989) respectively.

Direct visualization techniques

1. *Light microscopy.* The presence of many diseases in plant parts can be detected using various forms of light microscopy to visualize both disease symptoms in the host and signs of the pathogenic organism itself. In many cases the organism can be identified in this way. The procedure is quick and easy, but cannot reliably detect the smallest organisms such as viruses. The technique is particularly suited for the detection of fungi (Wilson, Clement and Kaiser, 1991) and in some cases for mollicutes using darkfield microscopy or fluorescent stains (Markham, 1988; Hiruki, 1988) or for particular virus inclusions (Christie and Edwardson, 1977).

2. *Electron microscopy.* The presence of many diseases can be detected in plant parts using various forms of electron microscopy to visualize both detailed disease symptoms in the host and signs of the pathogenic microorganisms themselves. These procedures can be useful to characterize a disease or its causal organism, but are unsuited for routine screening of the whole plant because of the very small parts which must be examined serially.

Although fungi can be detected by forms of electron microscopy, these techniques are more commonly used for diseases caused by bacteria, e.g. Sumatra disease of Cloves (Bennett, Jones and Hunt, 1987), by mollicutes (Markham, 1988) and especially by viruses (Baker, Ramsdell and Gillett, 1985; Christie and Edwardson, 1977; Cooper *et al.*, 1984; Roberts, 1986).

3. *X-ray radiography.* This non-destructive procedure is particularly suited to the examination of small, valuable collections of seeds, and has been used for many years to examine for insect infestation (Kobmoo and Skeates, 1986; Wadhi, 1983; Yates, 1974). For larger batches of seed, direct examination by cutting open may be preferable, however. More recently, attempts have been made to use it for detection of fungi (Vozzo, 1981; Kamra, 1974).

Biological and bioassay techniques

1. *Incubation on paper.* This procedure is particularly suited to the detection of fungi in plant parts and especially in seeds (Lange, 1986). It allows many fungi to grow on the host tissue so that they can be visualized and often identified. It is simple, quite quick, and easily carried out on many samples, and is thus well-suited to routine use for the detection of fungi in and on seeds. When combined with surface disinfection of the seed it can also indicate whether the fungi are superficial or are carried inside the seed.

2. *Incubation on agar.* Incubation on agar using various partial disinfection procedures combined with the use of antibiotics is particularly suited to the detection of fungi and bacteria in plant

parts (Dayan, 1986; Scortichini *et al.*, 1989), and especially in seeds (Lange, 1986). These techniques can also now be used to isolate some fastidious bacteria which require specific conditions for growth (e.g. Sumatra disease of cloves) and some mollicutes, using particular modifications to the basic procedure (Bennett, Jones and Hunt, 1987; Markham, 1988). Culturing the pathogen axenically in this way enables it to be better visualized and identified. The pathogen can also be subsequently characterized more fully and the degree of its pathogenicity determined.

3. *Transmission bioassay*. This procedure has been used for many years to detect the presence of submicroscopic pathogens in plant tissues. It involves the transfer of the microorganism to a range of indicator test plants either mechanically, by grafting, or by a range of types of vector (insects, mites, nematodes, dodder, etc.). The disease can then be characterized by evaluating which test-plants are affected and the symptoms caused on them. The procedure is particularly useful for viruses and mollicutes in plant tissues (Cooper, 1979; Hill, 1984; Markham, 1988; and Uyemoto, 1989), but is not sensitive enough for use with small seeds, and can take up to two years to complete.

Serological techniques

1. *Simple gel diffusion tests*. The formation of visible precipitin bands between antigens from pathogens, or from diseased tissues (containing the pathogen or substances produced by the pathogen), and specific antisera in gels have been used for many years to detect the presence of viruses (Uyemoto, 1989; Cooper, 1979; Uyemoto, Lowe, Schreader, Gubber and Kirkpatrick, 1989). Antisera in central wells on the dish are allowed to diffuse to meet antigens proceeding from wells around it. The technique can also be adapted to detect bacteria (Addy, 1986) or mollicutes (Sinha, 1986; 1988). It is not very sensitive, requiring appreciable quantities of specially prepared extracts, but can be made more sensitive and quantitative by the use of photometers.

2. *Agglutination tests*. Simple rapid agglutination tests of various types have been used to detect and identify plant viruses (Walkey, Lyons and Taylor, 1992). These have used particles such as chloroplasts, red blood cells, bacteria, latex, etc., to absorb specific polyclonal antibodies or viral antigens. These antibody-particle or antigen-particle combinations then agglutinate in the presence of particular viruses in crude sap extracts. The virobacterial agglutination test developed by Walkey *et al.* (1992) and other agglutination assays are claimed to be more sensitive and simpler to use than transmission bioassays and direct ELISA tests.

3. *Immunosorbent assays*. Many variations of immunosorbent assay procedures have been developed in recent years, with some being tested for a range of practical applications (Kulik, 1984). The procedures vary considerably in sensitivity, complexity and cost; some have become more used than others. Diffusion of antisera and antigens occurs on a sorbent and an enzyme is used to give a colour reaction. The direct enzyme-linked immunosorbent assay (ELISA) is most widely used in seed health testing (Lange, 1986), but indirect ELISA, dot-blot ELISA and others also have potential for this purpose (Lange, 1986; Barnett, 1986; Clark and Bar-Joseph, 1984; Sinha, 1988; Cooper *et al.*, 1984). These assays are used in the analysis of seeds and vegetative tissues particularly for viruses (Cooper *et al.*, 1984; Maury and Khetarpal, 1989), but they can also be used for bacteria (Lange, 1986; Mazarei, Hajimorad and Kerr, 1992) and mollicutes (Sinha, 1988; Markham, 1988).

The procedures can be made more sensitive and pathogen specific by the use of monoclonal antibodies. Development of these antibodies is time consuming and expensive, but the assays resulting can be very useful, particularly for fungi, with potential for use in field diagnostic kits (Van Regenmortel, 1986; Bossi and Dewey, 1992; Dewey, 1992; Dewey, MacDonald and Phillips, 1989; Hampton, Ball and De Boer, 1990). Immunosorbent assays can also be made more sensitive by the use of electron microscopy (Immunosorbent Electron Microscopy) (ISEM). This technique involves particle trapping and is very suitable for seed health testing (Lange, 1986); it has been used to detect bacteria, mollicutes and viruses in batches of seed or inside individual insects (Sinha, 1988; Markham, 1988; Milne, 1986; Baker, Ramsdell and Gillett, 1985; Brlanski, Lee, Timmer, Purciful and Rajn, 1992). However, closely-related organisms may not be separable using immunosorbent assays (Sinha, 1988).

Biochemical techniques

1. *Specific DNA hybridization*. Plant virologists employ variations of the DNA hybridization procedure in which a solid phase, such as nitrocellulose filters, is used (Markham, 1988; Morris, 1990). The most popular procedures include the 'Dot-blot' and 'Spot-blot' techniques and usually use virus-specific DNA probes produced by DNA hybridization (Barnett, 1986). The tests can be extremely sensitive and specific, and can utilize minute amounts of test materials (Markham, 1988). The tests have been utilized to detect mollicutes, viruses and viroids (Uyemoto *et al.*, 1989; Uyemoto, 1989; Markham, 1988; Barnett, 1986; Kirkpatrick, Stenger, Morris and Purcell, 1987; Hull, 1984, 1986; Owens and Diener, 1984) as they do not depend on the presence of an external protein coat.

2. *PCR assays*. These procedures do not employ specific probes and therefore can detect the presence of many microorganisms, including new ones (Barnett, 1986; Dodds, 1986; Dodds, Morris and Jordan, 1984). Non radioactive techniques which depend on the polymerase chain reaction (PCR), such as the random amplified polymorphic DNA assay (RAPD), detect dominant markers giving information on whether they are present or absent. The latter procedure has become widely employed recently to detect and identify a wide range of plants and microorganisms. Radioactive techniques using PCR procedures are also employed in recent applications such as 'tissue blot' systems.

SEED HEALTH TESTING AND PLANT QUARANTINE

Although dispersal of pests and diseases may eventually take place to all parts of the world where their host plants are widely grown (Gregory, 1979) either by natural means or by human agency, the longer the process can be delayed the better (Ivory, 1984). Consequently, most governments have enacted legislation to protect their own important crop plant species against the early introduction of alien pests. This legislation, whether based on a local, a national, or an international level, is now drawn up under the International Plant Protection Convention and is designed to control the movement of all plant materials, including that of forest trees, together with their packing, and thus the pests and diseases contained within or attached to them (Kahn, 1977). Regulations controlling the movement of forest tree germplasm between regions, or countries, usually contain a prohibition of plants with attached soil, and require all vegetative materials to be transferred through quarantine stations or under permit with specified inspections and treatments. Tree seed can often be imported without difficulty following inspection and treatment with pesticides. Tissue cultures are not always disease free despite the fact that disease is usually apparent quickly (Kahn, 1979); they are thus treated as vegetative materials and can be subject to quarantine for detection of viruses and other latent systemic pests (Ivory, 1984).

Plant health regulations usually entail inspection and certification at the point of export, and similar inspection and possibly quarantine at the point of import (Raychaudhuri and Verma, 1990) with the major onus falling on the importing authorities (Hewitt and Chiarappa, 1977; Anon, 1983). These measures, or their implementation, are often inadequate to detect all pests in vegetative or seed materials, particularly when the transfers are carried out on a commercial scale (Neergard, 1977; Kamra, 1990). Transfers of smaller quantities of germplasm for scientific purposes can also pose dangers, but safeguards can be employed to counter these risks (Singh and Rana, 1990; Anon 1991, 1989; Diekmann, 1988; Fassil-Kibebew, 1987; Kahn, 1983).

The development of rapid, reliable pest detection systems should enable inspections to be more frequent and thorough, both at the point of origin (to detect most organisms which might be carried with the germplasm) and at the point of entry (to provide efficient detection of known dangerous quarantine organisms).

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