UNIVERSITI PUTRA MALAYSIA

STUDIES OF TWO SEED-BORNE FUNGI OF SOME MALAYSIAN FOREST TREE SPECIES

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by

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This thesis attached hereto, entitled "Studies of two seed-borne fungi of some Malaysian forest tree species" prepared and submitted by Lee Su See in partial fulfilment of the requirements for the degree of Master of Science (Forestry), is hereby accepted.

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ABSTRACT

Twenty-six species of saprophytic fungi and ten species of potentially pathogenic fungi were isolated from seeds of five indigenous and three introduced forest tree species. Isolation of the fungi using both the blotter and the agar plate methods was carried out to ensure the isolation of as many species as possible from each seed sample. Six new species of fungi not previously recorded in Malaysia were discovered. They were *Beltraniella nilgrica* Pirozynski & Patil, *Chaetomium trilaterale* Chivers, *Cylindrocladium scoparium* Morgan, *Cryptodiaporthe* sp., *Gliocephalotrichum simplex* (J. Meyer) Wiley & Simmons and *Gliocladium* sp.

Various factors affecting the growth of two fungi - *Cylindrocladium scoparium* Morgan, a new species recorded in Malaysia which is potentially pathogenic, and *Pestalotiopsis versicolor* (Speg.) Steyaert, one of the most commonly occurring saprophytes - were investigated. These factors were the effect of nutrients, light, temperature, pH and various vitamins. Suitable culture conditions were developed for both fungi. Seedling inoculation tests showed that *Cylindrocladium scoparium* was pathogenic to seedlings of *Dipterocarpus grandiflorus* Blanco, a species indigenous to Malaysian forests, and *Pinus caribaea* Mor., a species of tropical pine introduced into and planted in this country. An investigation into some of the enzymes produced by the two fungi was also carried out to obtain a better understanding of the roles of *C. scoparium* and *P. versicolor* in seed deterioration and pathogenicity.

INTRODUCTION

Projections of timber supply and demand made by the Forest Department of Peninsular Malaysia have shown Peninsular Malaysia will be facing a timber shortage in the year 1995. To overcome the anticipated timber shortage and to achieve self-sufficiency in timber supply, the country is currently involved in reforesting logged over areas as well as embarking upon a programme of Compensatory Forest Plantations. With the increase in acreage of man-made forests and plantations in Peninsular Malaysia, the incidence of pest and disease problems will be of increasing concern. Research conducted in other countries has shown that although the incidence of major pest and disease problems is relatively low in undisturbed mixed forests, a large number of these problems occur in man-made forests. In India it was found that when indigenous species were raised in plantations, heavy losses occurred due to indigenous diseases. An excellent example is seen in Central and South America - the rubber tree never succeeded in plantations in its indigenous home due to the presence of the native leaf blight fungus, *Dothidella ulei*. Exotics on the other hand may be exposed to two hazards. They may be threatened by a pathogen inadvertently introduced which under favourable local climatic conditions may become epidemic. The exotic may also be attacked by an indigenous pathogen to which it possesses no resistance e.g. mortality of eucalypts in parts of India due to *Corticum salmonicolor*.

There is no doubt that the forest supports a varied community of potential pathogens which are capable of causing tree mortality, heart-rot and other defects which could degrade and reduce the volume of merchantable timber. Many of these potential pathogens may be seed-borne, but no efforts have been made so far in Malaysia to identify them. Since most of the forest trees grown in this country are propagated from seeds, it is important to know the fungi which are associated with the seeds and the impact these fungi may have no the developing seedlings or trees in the nurseries and in the field.

The purpose of this study was to identify the predominant fungi associated with seeds of selected tropical forest trees (*Shorea acuminata*, *Sh. materialis*, *Sh. singkawang*, *Sh. talura*, *Horsfeldia* sp., *Gmelina arborea*, *Leucaena leucocephala* and *Maesopsis eminii*). Two of these fungi, *Cylindrocladium scoparium* Morgan and *Pestalotiopsis versicolor* (Speg.) Steyaert were investigated in greater detail in an effort to better understand their physiology and capability of causing diseases. This study also hopes to elucidate the culture requirements of these two fungi and their enzymic capabilities.

It is anticipated that the results presented here will contribute new knowledge about seed-borne fungi of some tropical forest tree species which is lacking in Malaysia.

1. REVIEW OF LITERATURE

1.1 Seed-borne fungi of forest tree species

In the tropics seed science has hardly touched ground and seed testing is just in its developmental stage (Sevilla, 1977). This is probably why tropical seed species have been under-represented in the ISTA (International Seed Testing Association) rules (Buszewicz, 1978). The situation is very different in the temperate regions where seed testing has become an established practice for most seeds of economic importance, following standard rules and guidelines laid out by the International Seed Testing Association (ISTA, 1966, 1976).

The direct impact of fungi on seed is considerable. Many fungi are serious parasites of seed primordia and maturing seeds and reduce yields of seeds qualitatively and quantitatively. Fungi on seeds may be divided into "field fungi" i.e. those which invade seeds as they are developing on the plants in the field or after they have matured but before they are threshed; and "storage fungi" i.e. those which grow on stored products (Christensen and Kaufmann, 1965). "Field fungi" may be pathogens or saprophytes, common species being *Alternaria tenuis*, *Cladosporium herbarum*, *Curvularia* spp., *Epicoccum purpurascens* and *Fusarium* spp. while most of the storage flora are species of *Aspergillus* and *Penicillium* which can grow without free water and on media of high osmotic pressure (Neergaard, 1977; Pugh, 1972).

An extensive list of pathogenic seed-borne fungi has been compiled by Noble and Richardson (1968) covering 72 families of host plants, most of which are agricultural crops. To this list may be added a range of seed-borne fungi surveyed by Neergaard (1977). However most of the host plant species involved are agricultural crops and the list for timber or forest trees especially the angiosperms is rather limited. There is however a good record of fungi associated with seeds of gymnospermous forest/timber trees in the temperate regions. Shea (1960) observed that fungi, mainly species of *Penicillium* and *Aspergillus* reduced the viability and yield of Douglas-fir seeds during cold storage of cones before processing, subsequently contributing to seed decay and damping-off in the field. In later studies Bloomberg (1966, 1969) recorded *Gliocladium, Trichoderma, Trichothecium, Cephalosporium* and *Pullularia* from extracted seeds of Douglas-fir cones. Noble and Richardson (1968) have included in their list of pathogenic seed-borne fungi the species which are pathogenic to the following gymnospermous forest/timber trees - *Abies* spp., *Araucaria excelsa, Chamaecyparis* sp., *Coniferae, Gingko biloba, Juniperus virginiana, Larix* spp., *Libocedrus decurrens, Picea* spp., *Pinus* spp., *Pseudotsuga menziesii* and *Tsuga heterophylla.*

There have also been reports of seed-borne fungi of gymnosperms from countries in the Far East. Yamamoto *et. al.* (1966) have isolated and identified *Chaetomella, Trichoderma* and *Alternaria* from seeds of *Pinus densiflora* and *Pinus thunbergii.* In Japan *Picea* spp. have been known to be affected by cone rust in seed orchards (Faulkner, 1975). Studies have also been carried out to identify the microflora of coniferous seeds in Taiwan (Jong and Chen, 1964).

The record of seed-borne fungi of angiospermous tree species is however quite scanty in view of the enormous number of species. Fridrich *et. al.* (1972) isolated 30 genera of fungi from the fruits and seeds of *Betula alleghaniensis*, *B. papyrifera*, *Acer saccharum*, *A. saccharinum* and *Fraxinus americana*. They found that most of the fungi were probably saprophytic but did note that some could be pathogens. Golam *et. al.* (1971) frequently isolated members of the genera *Alternaria*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Pestalotia*, *Peyronellaea*, *Phoma*, *Phomopsis* and *Xylaria* from achenes of Sycamore (*Platanus occidentalis*) and observed that these fungi are similar to the "field fungi" reported from seeds of wheat, corn and barley. Many of the above fungi have also been reported from seeds of oak (Shea, 1960; Urosevic, 1962) and other hardwoods (Fridrich *et. al.*, 1972; Kozlowska, 1968 cited in Golam *et. al.* 1971, and Pugh, 1972). The seed-borne diseases of the following angiosperms have also been recorded by Noble and Richardson (1968) - *Acer* spp., *Alnus* spp., *Betula* spp., *Castanea* spp., *Fraxinus* spp., *Hevea braziliensis*, *Juglands* spp., *Nothofagus* spp.,*Quercus* spp. and *Ulmus* spp.

Similarly in Malaysia forest pathology research has concentrated on the gymnospermous tree species even though the angiospermous hardwoods form the mainstay of the country's timber resources. Most research thus far has been focused on diseases of the fast growing gymnospermous species. Freezaillah and Low

(1966) observed the outbreak of a fungal disease caused by *Pestalotia* sp. in potted Pine seedlings. This problem was however overcome by a combined treatment of fertilizer and fungicide applications and effective prevention was obtained when only fertilizer was applied before the onset of the disease. The pathological problems of fast growing conifers in Malaysia have been well investigated by Ivory (1972, 1973, 1975a, 1975b). He found that *Pinus* spp. in Malaysia are affected to varying extents by several diseases, the most important of which is the "brown needle disease" (BND) of *P. caribaea* and *P. merkusii*. Twelve fungi have been identified to be associated with BND at various times. Lim and Anthony (1970) reported *Glomerella cingulata* (Stonem.) Spauld & Schrenk, one of the twelve above as the cause of BND in Malaysia but this was found to be true only when plants were kept at high humidity and low temperatures for about two weeks. Ivory (1973) also noticed a second foliage and shoot disease sometimes occuring on old *Pinus* spp. seedlings in the nursery and on young plants in the field caused by *Cylindrocladium pteridis* Wolf. Other forest tree species which have been studied with respect to pathological problems include *Araucaria cunninghamii* (Griffiths, 1966; Ivory, 1972, 1975a), *A. hunstenii*, *Agathis dammara*, *A. macrophylla* (Ivory, 1972, 1975a) and *Eucalyptus deglupta* (Ivory, 1975a).

Records of some fungi associated with diseases of some other tropical forest and forest plantation trees, both angiosperms and gymnosperms can be found in Bakshi *et. al.* (1972), Browne (1968) and Singh (1973). Hong (1976) has recorded two fungi from two local angiospermous forest trees. He observed *Colletotrichum gloeosporioides* as being associated with leaf necrosis of *Shorea* spp. and *Cylindrocladium* spp. occassionally causing the death of *Dryobalanops aromatica* seedlings.

It is seen however that most of the references to the pathology of tropical species make little or no mention at all of forest tree seed pathology. Very limited research has been carried out in this field and there is little documented data. There is however some record of seed-borne fungi from research carried out at the Forest Research Institute, Kepong, Selangor, Malaysia. Hong (1974) frequently found a *Penicillium* sp. associated with seeds of *Araucaria cunninghamii* and *A. hunstenii* which often spread to the tips of the cotyledons and plumules causing the seedlings to die on germination. *Cylindrocladium* sp. has been recorded to occur on seeds of *Shorea assamica, S. ovalis, S. platyclados* and *Dryobalanops aromatica*. These seeds have also been found to harbour species of *Pestalotiopsis* and *Penicillium* (Hong, 1976). The same author also recorded a basidiomycete, *Schizophyllum commune* from seeds of *Shorea dasiphylla, S. assamica* and *S. platyclados*. In India *Aspergillus niger* has been found to be associated with stored seeds of *Shorea robusta* but protection against the fungus is afforded by the use of *Eucalyptus* hybrid oil (Singh *et. al.,* 1979). A fungus, probably *Fusarium* sp. has been found on seeds of *Leucaena leucocephala* in the Philippines as a result of a beetle attack whereby the fungus enters the pod via the oviposition hole of the beetle (Viado, 1979).

More recently Maury-Lechon *et. al.* (1980) working in Kalimantan observed that fungal attack accounted for a significant loss in viability of collected Dipterocarp seeds. Yap (1980) states that the inherent high moisture content of Dipterocarp seeds enhances pathogenic attacks especially by fungi. However, no documentation of the fungi species was made and further studies are necessary.

The number of tropical forest tree species considered so far represents only a very small portion of the large number of species found in the tropics. In Peninsular Malaysia for example, it has been estimated that there are about 7500 species of seed plants of which only about 3000 species are named (Whitmore, 1972) and of which only about 402 species are commercially valuable (Kochumenn, 1973). Thus it is seen that knowledge of the fungi associated with and causing injury to seeds and seedlings of angiospermous trees is far from complete.

1.2 Isolation techniques for fungi from seeds

The importance of seed-borne diseases was not generally recognised until 1927 when a Russian worker, Zybina examined 300 samples of flax seeds from all parts of the world for the presence of micro-organisms (Porter, 1949). Between 1926 and 1930 Doyer examined commercial seed lots of flax, peas, beet, beans, wheat and barley and in 1938, she published a useful handbook which gave the general classification of seed-borne organisms, methods for their detection and control and a list of the host plants and associated organisms together with illustrations of their symptoms and morphology. Since then much more research has been carried out and there now exists standard methods for isolation and detection of fungi from seeds.

It is known that fungi, bacteria and viruses are seed-borne and that of these, fungi are the most common. However, fungi may be pathogenic to plants being storage fungi, while others are pathogenic or symbiotic (Limonard, 1968). There are four main methods for isolating and detecting fungi from seeds; viz:-

1) Examination of dry seeds
2) Examination of material removed from seeds by washing
3) Examination after incubation
4) Examination of growing plants (ISTA, 1966)

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

1.2.1 Examination of dry seeds

Dry seed is examined for impurities, classified partly as 'inert matter' in the International Rules for Seed Testing (1966) such as plant debris, sclerotia, ergots, galls, smut balls etc. and for discolorations and malformations due to pathogenic conditions. The seed is examined under a low power microscope with magnifications of up to 50-60 times where fungi can sometimes be identified by fruiting bodies such as acervuli and pycnidia.

Attention should also be paid to chaff, straw or other inert matter in which pathogenic fungi may also be present (De Tempe & Binnerts, 1979).

Brown spots on seeds of legumes often indicate *Colletotrichum lindemuthianum* and other anthracnose fungi, or *Ascochyta pisi*, *Mycosphaerella pinodes* and other parasites in pea and pycnidia of *Septoria* and *Phoma betae* are commonly seen on dry host seeds (Neergaard, 1977). Storage fungi in cereals may be detected by the darkening of the germ (Christensen, 1957). The identification of some infections such as *Ascochyta pisi* in peas and *Stemphylium botryosum* Wallr. in *Phaseolus* beans is facilitated by fluorescence of the spots in near UV light (Neergaard, 1977; De Tempe & Binnerts, 1979) however Anselme and Champion (1962) state that it is not a very accurate health testing method.

Although direct inspection of dry seeds provides quick information and does not require much equipment, it can however only detect few diseases (Limonard, 1968). Neergaard (1977) states that although anthracnose fungi and *Ascochyta* spp. of Leguminosae produce symptoms on the seed, incubation tests are imperative to provide full information on the development of the fungi. Moreover symptoms may not be produced on the dry seeds if the incidence of that fungi is weak (Naumova, 1972).

1.2.2 Examination of material removed from seeds by washing

This method has been of great practical importance for a long time (Heald, 1921) and is useful for the detection of fungus spores, hyphae, stem nematodes, etc. which are removed from seeds by vigorously shaking 100 or more seeds in water plus a wetting agent or in alcohol. The fluid is then filtered, centrifuged or evaporated to a few drops before examination (ISTA, 1966). The concentrated remainder may also be diluted by a known dilution factor and examined under the microscope using a haemocytometer (Neergaard, 1977). The spores may also be collected on filter paper which is then cleared so that the spores may be counted under the microscope (Gentner, 1929 cited in Neergaard, 1977; Kietreiber, 1976 cited in De Tempe & Binnerts, 1979). Other procedures have been described by De Tempe (1963 a) and Naumova (1972). The amount of spores in the suspension may also be determined by turbidimetry using a spectrophotometer (Neergaard, 1977).

This technique is widely used for quantitative determination of the spore load of smut fungi in cereals and for determination of any fungi adhering to the seed surface such as *Alternaria, Stemphylium, Drechslera, Fusarium* and others (Naumova, 1972; Neergaard, 1977). However it is less reliable in the case of *Ustilago avenae* and *U. hordei* (Pers.) Lagerh., in which the spores partly penetrate between the glumes and the caryopsis (Gassner, 1953 cited in De Tempe and Binnerts, 1979) although these spores are most likely to infect the seedlings. Mahdi (1968) found that this method gave the highest percentage of *Pyricularia oryzae* infected rice seed samples although it did not indicate the viability of the spores. While Mahdi (1968) recommends the shaking method over the blotter method for routine testing of this rice disease because of its simplicity and