PATHOLOGICAL ASPECTS OF SEED-BORNE MYCOFLORA OF *Pinus caribaea* Morelet var. *hondurensis*.

By

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A THESIS IN THE DEPARTMENT OF AGRICULTURAL BIOLOGY
SUBMITTED TO THE FACULTY OF AGRICULTURE AND FORESTRY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
OF THE
UNIVERSITY OF IBADAN

ABSTRACT

A survey of the mycoflora of three seed lots of Pinus caribaea var. hondurensis was carried out using the incubation tests for the detection of seed-borne fungi on agricultural seeds. The seed-borne fungi identified were: Acromonium sp., Aspergillus flavus, A. fumigatus, A. niger, A. tamarii, Botrytis sp., Botryodiplodia theobromae, Chaetomium funicola, C. globosum, C. indicum, Cladosporium oxysporum, Dendrophoma sp., Fusarium equiseti, F. moniliforme var. intermedium, Gliocladium roseum, Macrophoma spp., Mucor spp., Paecilomyces variotii, Penicillium citrinum, P. cyclopium, Pestalotiopsis spp., Phialophora fastigiata, Phomopsis occulta., Rhizopus sp. and Trichoderma sp.

Seed-debris (pieces of cones and twigs) and stones also carried a large proportion of these pathogenic and saprophytic fungi. Using scanning electron microscope, no propagules of obligate parasitic pathogens were seen on the seed coat but spores and mycelia of some facultative pathogens were observed. Most of the seed-borne fungi were carried externally on the testa.

Pinus caribaea var. hondurensis seeds inoculated with spore suspension and mycelial discs of the following

fungi: Aspergillus niger, Fusarium moniliforme var. intermedium and F. equiseti had drastic reduction in germination. F. moniliforme var. intermedium and F. equiseti caused wilting and sudden death (damping-off) in 3 week and 3 month-old inoculated seedlings (20-60% mortality) 7 days after inoculation. Inoculated stems showed disintegration of cell-walls, parenchyma cells and fibres. Aspergillus niger had no effect on inoculated seedlings while Phialophora fastigiata had no effect on both the inoculated seeds and seedlings.

Seeds which were surface sterilized for 5 minutes in 1% sodium hypochlorite and planted in autoclaved sterilized soil gave a germination percentage of 42 while seeds which were not surface sterilized but planted in sterilized soil gave 20%. Damping-off and wilting were observed in 10-12% of the seedlings which emerged from the two treatments 5-30 days after germination. Botryodiplodia theobromae, Fusarium moniliforme var. intermedium, Rhizoctonia spp. and Rhizopus spp. were isolated from the diseased seedlings.

The effect of three fungicides: Benlate, Captan and Thiram in controlling the growth of the frequently occurring seed-borne fungi (Aspergillus niger, Fusarium moniliforme var. intermedium and Phialophora fastigiata)

was studied *in vitro*. Only Benlate at concentrations of 1 ppm and above inhibited the growth of the three fungi. Thiram inhibited the growth of *Aspergillus niger* and *Phialophora fastigiata* at 20 and 400 ppm respectively while Captan inhibited the growth of *Aspergillus niger* at 40 ppm.

ACKNOWLEDGEMENT

I am thoroughly indebted to my supervisor Professor M.O. Adeniji and the other members of my supervisory committee: Drs. E.J.A. Ekpo, J.A. Odebiyi and M.A. Amakiri for their interest, support and constructive criticism.

Sincere thanks to the University of Ibadan and the British Council for the Inter-University Council (I.U.C.) grant which enabled me to visit the University of Oxford, Oxford Forestry Institute, U.K., Commonwealth Mycological Institute, Kew, Forestry Commission, Aliceholt and other parts of the United Kingdom where some aspects of this study were carried out. I thank most sincerely Drs. Ray Pearce, Mike Ivory and John Gibbs for the use of their laboratories, libraries and all necessary facilities; the technical and library staff of Oxford Forestry Institute; my colleagues there, especially Messrs Tope Popoola, Fortunato Garza Ocanas, Dr. Tony Ofong and Miss C.D. Toscano; Dr. J. Parker of Forestry Commission, Aliceholt, U.K.; Dr. R.G. Pawsey (formerly of Aberdeen University, U.K.); Dr. T.F. Preece, University of Leeds, U.K. and Prof. P.T. Onesirosan, Obafemi Awolowo University, for their contributions.

I am grateful to the Director and staff of Forestry Research Institute of Nigeria, Ibadan for providing the seeds, laboratory facilities and necessary assistance for this study. Particular here are Drs. M.A. Odeyinde, M.O. Akanbi, D.O. Ladipo, O.O. Okoro, Mrs. A.F. Somade, Messrs Dada, K.D. Obisesan, J.O. Eyenike and S.A. Akanni.

My appreciation also goes to:- Prof. O.F. Esuruoso, the initial supervisor of this study for his keen interest, guidance and assistance; Profs. R.O. Adegboye, S.K. Adeyoju, D.U.U. Okali, E.E. Enabor and L.C. Nwoboshi for their valuable contributions towards the accomplishment of this study; Drs. F.A. Akinsanmi, A.E. Falaye, J.S.A. Osho, J.A. Okojie, S.K. Sanwo, S.O. Bada, A.I. Ayodele, M.O. Asaolu, M.O. Sanni, Sola Ogunyemi, F.O. Akintola, Messrs O.K. Sokunbi, S.D. Owoyomi and all staff of the Departments of Agricultural Biology and Forest Resources Management for their useful suggestions and encouragement; Dr. (Mrs.) Faparusi of the Computer Centre, University of Ibadan, for her help in the statistical analysis of the data; my friends and colleagues for their interest and moral support; Mrs. A.F. Ogunsuyi and the secretarial staff of the Department of Forest Resources Management for typing the manuscripts.

A very special and profound gratitude goes to my husband, children and relations for their encouragement and support throughout the duration of this study.

Finally, I thank the Almighty God for sparing my life and seeing me through the completion of this study.

CERTIFICATION

I certify that this work was carried out by Mrs. A.O. Adegeye of the Department of Agricultural Biology, University of Ibadan.

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

The rapidly increasing demand for wood production in Nigeria will be met largely by the creation of plantations in both the lowland moist forest and the savanna zones (F.D.F., 1982). Enabor (1976) noted that the total wood consumption requirements (fuelwood, polewood, sawnwood, wood-based panels, paper and paper board) in Nigeria rose from 58.12 million m^3r (r = roundwood) in 1971 to 89.57 million m^3r in 1985 and will rise to 122.23 million m^3r in the year 2000. It was predicted that by 1995, the natural forest of Nigeria will be unable to meet the demands for industrial roundwood and that shortages will be enormous. The country's ultimate requirement to meet this shortfall is estimated to be 1 million hectares in the moist forest zone and 0.8 million hectares in the Guinea savanna (FAO, 1979). The area planted in Nigeria to trees increased from 7,000 hectares in 1953 to 13,000 hectares in 1963 (Iyamabo, 1967), 93,000 hectares in 1973 (Anon, 1975) and 150,000 hectares in 1980 (World Bank, 1986). All these gigantic plantation schemes will require large quantities of seeds of the best quality at the right time (FAO,

1955). Seeds for this afforestation programme will require collection and storage until the right conditions for growth exist.

The FAO seminar on Tropical Pines in Mexico in 1960 (I.N.I.F., 1962) created an awareness in Tropical countries in the value of pine species. In Nigeria, Pinus caribaea Morelet and P. oocarpa Schiede have been successfully used in the re-forestation of Guinea savanna (Kemp, 1969) and derived savanna (Ekeke and Ojo, 1981). The tropical pine, Pinus caribaea Morelet variety hondurensis Barret and Golfari is a species of great versatility and variability. It is capable of producing any particular quality of wood given the right silvicultural and breeding techniques (Plumptre, 1984). It is used as a source of softwood material for augmenting the world supplies of pulp (Plumptre, 1984; Okoro, 1986) and also for the afforestation of degraded or naturally infertile sites (Greaves, 1980).

The Commonwealth Forestry Institute at Oxford with its unit of Tropical Silviculture, supplies pine seeds to Nigeria and other desiring countries (Lamb, 1973; Greaves, 1978). Eventually it is hoped that the demand for pine seeds will be met from local sources. Unfortunately, a

lot of difficulties are involved in obtaining adequate quantities of seeds for large-scale establishment of P. caribaea var. hondurensis. Such difficulties have been linked with apparent lack of flowering, poor seed production which occurs when the species is grown as an exotic and diseases (Nikles et al., 1978; Okoro and Okali, 1987). Seed-borne fungi such as Pestalotia spp. and Fusarium roseum "Gibbosum" have been isolated from seeds of P. taeda L. (Mason and Van Arsdel, 1978). These seed-borne organisms were found to reduce seed germination and seedling growth. Pawuk (1978) also confirmed these observations with F. moniliforme Sheld, F. oxysporum Schlecht and F. solani (Mart.) Sacc. which he isolated from some long leaf pine seeds. Rees (1983) also showed that Botryodiplodia theobromae Pat. was a seed-borne pathogen of P. caribaea.

1.2 Justification for Study

Since 1940, the Federal and State Governments of Nigeria through their various forestry departments, have concentrated their efforts on the establishment of fast-growing exotic tree species throughout the country. The species include Pinus caribaea (Morelet). Nigeria still

continues to import pine seeds because the quantity of seeds produced by her pine plantation is not sufficient to meet her demand (Okoro, 1986).

The pulpwood plantation for the softwood requirement of Nigerian paper mills is estimated at 18,000 hectares per annum. The pine component is about 23.5% (4,430 hectares). Fourteen states have been identified for participating in the establishment of the pine components. Jebba has also planned additional 200 hectares annual planting. About 221.5 kg good seeds are required for this pine plantations annually. Seed procurement for the targeted annual planting will cost $31,010 (or ₦155,000) in foreign exchange (Okoro, 1988). The locally produced seeds have a low germination percentage of 6-48 depending on the collection site. All the pine seed requirement in the country has been met from importation except 0.325 kg collected once from Miango and Afaka in 1974. Hence, for sometime to come, we shall have to depend on imported pine seeds (Okoro, 1988).

Again, at the Forestry Research Institute, Ibadan, which is responsible for storing the various tree seeds and for raising of seedlings for the afforestation programmes, "mould-like" organisms are usually observed on

the imported and stored pine seeds during routine germination tests and such seeds show low germination percentage (Dada, personal communication). The low germination of the seeds therefore means that more seeds have to be sown per hectare and this is a waste of our resources (monetary and human). Also, if the mould-like organisms observed on the seeds in the germination tanks are carried to the nursery and the field, they could contaminate the soil during the normal silvicultural operations (prickling and watering) and act as loci of infection for the seedlings and mature plants. Therefore, efforts should be made at the earliest stage of planting operations to reduce seed-borne micro-organisms, especially fungi, which might lead to devastating diseases of pines. The work reported in this thesis was therefore initiated to examine, and as far as possible characterize the mycoflora of the imported pine seeds used in Nigeria afforestation programmes. The effect of this mycoflora on seed germination, seedling vigour and disease development has also been examined.

1.3 Objectives of the Study

The objectives of the study were:

(a) to isolate and identify the seed-borne fungi on *Pinus caribaea* var. *hondurensis* samples,

(b) to determine the location of the seed-borne mycoflora in/on the seeds through component plating of seed parts and scanning electron microscopy,

(c) to test the pathogenicity of some of the seed-borne fungi and

(d) to test *in vitro* the effect of some fungicides on the growth of some of these fungi.

CHAPTER TWO

LITERATURE REVIEW

2.1 Afforestation in Nigeria

Until the past few decades, the world's estimated 2,500 million hectares of closed forest, supplied the majority of forest products used by mankind (Evans, 1982; Kanowski, 1986). In the recent past, these products became increasingly concentrated in more intensely managed forests, for a variety of social and economic reasons such as: continuing exploitation of natural forests, controlling erosion and reducing dependency on imports (Wood, 1976; Evans, 1982 and Lanly, 1983). Much of this intensively managed forest is in the form of plantations, of which upwards of 100 million hectares have been established. About 30 million hectares of this lie between latitudes 27°N and S, which has been broadly defined as the tropical region (Evans, 1982). New establishment, which approximates 10 million hectares annually is concentrated within this region which includes Nigeria (Evans, 1982).

Nigeria lies between latitudes 4° 18'N and 14° 24'N, longitude 3° 00'E and 14° 30'E. The Nigerian main forest belt occupies the southern part of the country. Starting

from the coast is the mangrove forest which occupies about 12,783 km^2. Next is the swamp forest, 25,562 km^2, then the lowland forest proper, 95,372 km^2. The remaining part of the country, 849,496 km^2 is occupied by savanna vegetation. Islands of forest formations are also scattered all over the country in moist hollows, river valleys or hill tops in Niger, Kaduna, Plateau and Gongola States.

The lowland forest proper forms a continuous belt about 300-350 km wide from the west to the south east. This occupies about 10% of the land area of Nigeria. Only about 20% of this forest estate is under reservation (19,986 km^2 or 2% of total land area of Nigeria). The rest is community forest or "free area" (Bada, 1984). This forest area accounts for more than 96% of the total timber supply of the country. If immediate steps are not taken to regulate the rate of forest exploitation and conversion, the remaining pockets of forests in the free areas as well as the forest reserves may be exhausted before the year 2000 (Kio, 1978).

It is obvious from the above that Nigeria should prepare herself to provide a vastly increased volume of wood products by the year 2000 to meet the needs of the

population. In view of the growing demand for wood and wood products in Nigeria resulting from increasing population and rapid economic development, both the Federal and State governments have regarded Nigerian's 2% of productive forest area as very low. Steps have been taken to replenish the country's renewable forest reserves through intensive forest regeneration programmes (Oseni, 1977). These are aimed at producing timber for transmission poles, pulpwood for the pulp and paper mills, tannin material and gum arabic for the industries. Other objectives of the programmes are to provide shelterbelts/windbreak against encroaching desert and environmental degradation in the arid north of the country. These programmes involve establishing plantations through nurseries and hence the need for seeds. Within the last two to three decades, the need for tree seeds has increased considerably in Nigeria with the unprecedented expansion in afforestation programmes.

2.2 *Pinus caribaea* (Morelet): origin, distribution and biology

Pinus caribaea belongs to the division Spermatophyta, order Coniferae, family Pinaceae, genus *Pinus* (Mirov,

1967). Pines are conspicuous trees (rarely shrubs), either forming extensive forests or contrastingly admixed with broadleaf trees.

Pinus caribaea is widely distributed in Central America and the Caribbean, between 27° 25'N latitude in Grand Bahama and Great Abaco and 12° 13'N latitude near Bluefields on the East Coast of Nicaragua (Appendix I). The longitudinal range is from 71° 40'W on Caicos Islands to 89° 25'W at Poptun in Peten province of Guatemala. The tree grows from sea level up to an altitude of approximately 100 m (Lamb, 1973). In the northern half of its distribution Pinus caribaea is found mainly in warm, moist savanna climates with a dry winter period. In the southern parts of its distribution belt, particularly in Nicaragua, the species occur extensively in tropical savanna climates with a short dry season and a rainfall which is sufficiently high to support tropical rainforest (Luckhoff, 1964). Pines grow in areas with mean monthly rainfall less than 50 mm and mean monthly maximum temperature between 22.1 and 32°C. It is found on a very wide range of bedrock, usually on steep slopes. It performs best on well aerated soils, low in nutrient, with slightly acidic pH of 4 - 5.5. It is now the most

important lowland timber tree planted in tropical countries (Lamb, 1973). Three varieties, bahamensis, caribaea and hondurensis are recognized (Barrett and Golfari, 1962).

P. caribaea is a large pine reaching 45 m in height under the best conditions on the Mountain Pine Ridge in British Honduras and 1.35 m in diameter at breast height in Little Pine Ridge on the western slopes of the Mayan mountains in British Honduras (Mirov, 1967). Pines produce seeds at intervals of two, three or even more years. Seed-bearing capacity decreases with old age, but it does not cease entirely, and pines continue to produce seeds until their death. The life span of some pines, such as P. radiata D. Don. does not exceed 150 years while that of P. lambertiana Dougl reaches 600 years (Mirov, 1967).

Pine seeds are narrowly ovoid, about twice as long as broad, pointed at both ends, 3 angled, averaging less than 6 mm long, 3 mm wide, black, mottled grey or light brown, with a membraneous brown wing less than 20 mm long, sometimes fused with the seedcoat but usually becoming detached when moist (Lamb, 1973).

For plantation use, pines are generally raised from seeds. In Nigeria and several countries still using

expensive imported seeds, fine, pure, sterile sand in large wooden boxes or concrete sided beds are used for the pre-germination of seeds. The pre-germinated seeds are transferred to polythene pots 6-10 days after germination. The plants remain in the polythene pots in the nursery for 6-8 months from time of sowing till they are put out in the field as 20-30 cm transplants. NPK fertilizer and mycorrhizal fungus are added to the soil in the polythene pots (Lamb, 1973).

The first pine plot in Nigeria was laid down with Pinus caribaea var. hondurensis in 1961 at Miango in Jos, Plateau State (1,200 m altitude) (Iyamabo et al., 1972). These trees were 13 m high when 9 years old, fused needles were produced each dry season and normal needles during the wet season.

2.3 Seed-borne fungi on conifers

Several broad categories of fungi have been isolated from conifer seeds (Anderson, 1986a). There are species that cause decay and reduce germination of stored seeds, species that attack germinating seeds and seedlings, and other species that are more or less harmless. Some fungi usually regarded as harmless can cause serious losses

under conditions adverse for the seeds such as improper seed storage. In some cases, fungi that are normally considered to be saprophytes can cause decay of seeds, for instance Trichoderma (Urosevic, 1961). Rees (1983) working with the seeds of some Pinus spp. including P. caribaea, isolated 155 fungal species, of which 27 were plant pathogens. Pawuk (1978) recorded 4 to 20% infestation of long leaf pine seeds with Fusarium moniliforme, F. oxysporum, F. solani, F. roseum and F. tricinctum (Corda) Sacc. The author noted that F. roseum isolates reduced germination, F. moniliforme and F. roseum isolates increased the occurrence of post-emergence damping-off and led to reduced seedling growth. Mason and Van Arsdel (1978) also isolated various fungi from over 95% of seeds of Pinus taeda L. from some seed orchards in Texas. Such fungal isolates included Pestalotia spp., Fusarium roseum, F. tricinctum, Rhizopus arrhizus Fischer, Syncephalastrum racemosum Schroet and Hyalodendron spp. from seeds which were washed for 2 hours in a screened container with vigorously flowing tap water. Seeds surface sterilized in 30% hydrogen peroxide for 45 minutes yielded Geotrichum spp., Pestalotia spp., Curvularia spp., Aspergillus spp. and Penicillium spp. First year cones

produced Pestalotia spp., Gilmaniella spp. and Epicoccum nigrum L. while second year cones had F. roseum, F. trincinctum, F. oxysporum, F. moniliforme and Trichothecium spp. Therefore, the method employed in seed-borne fungus isolation and the age of the cones affected what type of organisms were obtained. They also established that 16 of their 61 isolates reduced germination and seedling performance. This study also confirmed an earlier study in Texas by Mason (1976) who isolated 15 fungal species and found that germination was reduced by F. moniliforme and Pestalotia sp.

The seeds of conifers can also become infected when in contact with the soil (Sutherland and Woods, 1978). These authors isolated the fungus Geniculodendron pyriforme Salt only from seeds of cones collected from the ground beneath Sitka Spruce. Singh (1981) also reported that seeds from unopened cones did not yield any fungi but those extracted from partly opened cones yielded a few species. He found that most seed-borne fungi were carried externally on the testa.

According to Bloomberg (1969), Douglas Fir seeds do not become diseased in the cones, whereas significant number of seeds from the cones may become diseased in

germination tanks. Also externally borne, and endophytic fungi are abundant in Douglas fir seeds after kiln extraction (Bloomberg, 1966). Many fungi are also observed during routine testing of conifer seeds, the most frequent being Penicillium spp. (Holmes and Buszewicz, 1953, 1954).

The seedcoat of most coniferous species is relatively thick and resistant to fungal invasion, but seeds are frequently slightly damaged during extraction and then become infected (Whittle, 1977; Gibson, 1957). An examination of the ability of surface-borne fungi to colonize damaged seed of Pinus patula Schlecht and Chamisso suggested that fungi harmless on the surface of pine seeds can become destructive once the seedcoat has become even slightly damaged (Gibson, 1957).

Anderson et al. (1984) observed internal seed infection by fungi in 3-88% of P. elliottii Engelm. seeds from some seed orchards in the United State of America. These researchers recorded pathogenic fungi such as F. moniliforme and Diplodia sp. and found that the occurrence of pathogenic fungi was about the same for healthy and unhealthy seeds, except for Diplodia, which was higher in unhealthy seeds. In the two-year survey, it was concluded

that occurrence of internal fungi was variable. The year of collection of seeds affected the types and numbers of fungi recorded. The isolated fungi caused flower, cone and seed losses in P. elliottii.

Up to the late 1970s very little work had been done on the seed microflora of tree species (Rees, 1983). Most studies have been in agriculture and horticulture where the effect of plant disease is obvious within one growing season (Neergaard, 1975 and Noble, 1951). Inglis (1980) used scanning electron microscope (SEM) to study fungi on asparagus seed. He found that the surface of an asparagus seed was rough and that Fusarium moniliforme was present externally in the natural crevices of the seed coat or in the cavities of seed damaged by asparagus beetle. Patton and Spear (1978) also used the SEM to study the development of germ tubes from conidia on the needle surface and in the stomatal antechamber of Scotch pine. The authors found that germ tubes usually grew appressed to the needle surface and followed contours of the epidermis. Also germ tube growth seemed to be directed specifically towards an individual stoma, and in the antechamber the germ tube usually increased in diameter, became thick walled, melanized, rugose in surface texture,

and irregular in general form and outline. Rees (1983) using the SEM was able to locate the spores of Botryodiplodia sp. on Pine seed coat.

2.4 Diseases of conifers

The most important fungal diseases of exotic pines are:

(i) "Damping-off", a disease complex which may cause considerable losses in the germination bed before and after seedling emergence. There are few projects for the establishment of exotic pine plantations that have not encountered losses from this disorder during the early stages of their development (Gibson, 1979). Ivory (1987) reported that pre-emergence damping-off is characterized by a wet rot of the radicle, while post-emergence damping-off occurs as a collapse of the stem tissues at soil level, causing the seedling to fall over and lie on the surface of the soil after which it dies and shrivels rapidly. The disease can spread rapidly, resulting in large patches of dead and dying plants in which the most recent deaths are at the outside of the patch. This pattern of spread is typical of a disease caused by soil-

borne agencies. The disease may also come from infected seeds (Ivory, 1987).

All forest species are susceptible, to a greater or lesser extent, to this disease. It is caused by a wide range of soil inhabiting fungal pathogens of which the most important are Phytophthora spp., Fusarium spp., Thanatephorus cucumeris (Frank) Donk (syn. Rhizoctonia solani Kuln) and Pythium spp.. All have a wide host range and survival is by means of chlamydospores, sclerotia or oospores (Baker, 1972; Bakshi, 1976; Gibson, 1979; Malone and Muskett, 1964).

Parker (1964) and Ofong (1982), reported damping off caused by Fusarium spp. as an important problem of the Nigerian forest nursery. The affected seedlings were mainly Pines and Eucalypts. In Tanzania, losses of 60-70% have been reported in pine nurseries as a result of damping off (Hocking, 1968a&b; Hocking et al., 1968; Hocking and Jaffer, 1969). These researchers showed that Fusarium spp. mainly F. oxysporum, F. solani, F. moniliforme and Thanatephorus cucumeris were the most frequently associated with diseased plants and were also the most virulent pathogens of a wide range of isolates. Sclerophoma pithyophila (Corda) Hohn. and Botryodiplodia

theobromae were also shown to be important pathogens, but Pythium acanthicum Dreschl and Curvularia tuberculata Jain were found to be only weakly pathogenic to P. caribaea.

Seedlings increase in resistance to damping off as they grow older and the tissues at the base of the stem become harder and more lignified with secondary thickening. Damping off can also cause considerable losses immediately after transplanting when bruising of the stem by handling and planting the seedling too deep can contribute to susceptibility (Hocking, 1968b). Spread of damping off pathogens can occur by transfer of infected soil, rain splash, watering or sometimes be seed-borne (Gibson, 1979).

(ii) Charcoal root rot or black root rot caused by Macrophoma phaseolina (Tassi) Goid. This was reported on Nigerian-grown pine by Gibson in 1979. The disease occurs in young plantations as well as nurseries and has been fully investigated in different countries outside Nigeria by Hodges (1962), Smith and Bega (1964), Smith and Krugman (1967) and Seymour (1969). The root cortex decays and becomes covered with a dark-brown crust of fungal mycelium. The aerial parts of the plants become stunted and discoloured and eventually wilt and die (Ivory, 1987).

(iii) Cercospora Needle Blight or Brown Needle Disease caused by Cercoseptoria pini-densiflorae (Hori et Nambu) Deighton (Syn. Cercospora pini-densiflorae Hori et Nambu). The symptoms first appear as pale green bands on the needles which quickly turn yellow, then brown and finally greyish brown. Eventually the whole needle may die and turn greyish with lines of minute sooty spots coincident with the stomata (Mulder and Gibson, 1972). Defoliation by Cercospora needle blight can cause significant reductions in seedling growth (Ivory, 1975).

(iv) "Terminal Crook" or "Gleosporium Terminal Crook" caused by Colletotrichum acutatum Simmonds f. sp. pinea Dingley and Gilmour. This is a nursery disease that may cause the death of very young seedlings (Gibson, 1979) and a severe stunting of older seedlings after transplanting in the nursery. The first sign of the disease in older seedlings is the death of needles in the terminal whorl from the base upwards with a turning over or "crooking" of the tip. Growth from the terminal bud ceases and the stem becomes thickened and rigid, particularly towards the tip. This results in stunted growth. Defoliation will also take place in diseased plants, but, unless they are very young, they will not die. Distribution of the disease in

the nursery is usually patchy but in severe attacks, the whole crop may be more or less uniformly damaged (Dingley and Gilmour, 1971).

(v) Cylindrocladium Shoot Blight caused by Cylindrocladium pteridis Wolf. (Syn. C. macrosporum Sherbakoff) affects Pinus caribaea var. hondurensis, P. oocarpa and P. Kesiya Royle ex Gord. This disease was first observed in West Malaysia (Ivory, 1973). First symptoms appear as a bright red-brown necrosis of a leading or secondary shoot and reddish brown sunken lesions on the foliage. Needle lesions lead to necrotic banding and the death of distal parts, after which the needle is cast. The disease has been observed in nurseries and young plantations in the USA (Sobers, 1968).

(vi) Diplodia Die-back or Whorl Canker caused by Diplodia pinea (Desm.) Kickx. syn. Botryodiplodia pinea (Desm.) Petrak, Macrophoma pinea (Desm.) Petrak and Syd. It was one of the first fungal pathogens identified as a cause of stem and foliage disease in exotic pine plantations in the Southern hemisphere. Bancroft (1911) reported that successful infection required wounded tissues. But elsewhere, Waterman (1943) and Chou (1976a&b) found that the fungus could invade unwounded

green shoot tissues or needles, eventually causing die-back. This fungus has been found to be seed-borne (Noble and Richardson, 1968). This probably has contributed to its widespread occurrence where pines have been planted as exotics.

(vii) Pitch Canker caused by Fusarium lateritium Nees emerd. Synder and Hansen f. sp. pini Hepting, syn. Gibberella baccata (Wallr.) Sacc. (perfect state). This affects species such as Pinus radiata, P. echinata Mill., P. elliottii, P. caribaea. The symptoms of the disease include sunken areas of bark (which is not shed) on the main stem accompanied by excessive resin exudation and resin-soaking of the wood under the cankered areas (Bethune and Hepting, 1963). These can lead to deformation and killing of young saplings by girdling.

(viii) Dothistroma needle-blight (Red-band or Red-spot Disease) caused by Mycosphaerella pini E. Rostrup apud Munk (Syn. Scirrhia pini Funk and Parker). This is probably the most important foliage disease of exotic tropical pines (Ivory, 1987). The symptoms are red-brown necrosis of needles and subsequent needle cast. The disease can severely stunt or kill nursery plants and young trees. Conidia are the most important means of

spread. These are released from infected needles during light rain and misty conditions. The spores may be carried for miles in the clouds (Gibson, 1979).

(ix) Fomes Root-Rot, 'Fomes annosus' caused by Heterobasidion annosus (Syn. Trametes radiciperda Hartig, Fomes annosus (Fr.) Cooke, Polyporus annosus Fr.) This is probably the most economically important root pathogen of forest crops. It is probably the most-damaging conifer disease in Europe, North and Central America. It causes stem decay, loss of growth and tree mortality (Ivory, 1987). It is not reported in tropical plantations of exotic pines (Gibson, 1979).

(x) Brown Root Rot caused by Phellinus noxius (Corner) G.H. Gunn. The disease affects Pinus caribaea var. hondurensis and many other species. It forms a brown mycelial mat on the outersurface of infected roots. This mat eventually turns black. The root tissues are at first discoloured red-brown with zigzag lines and later become dry, friable with sheets of mycelium and pockets filled with brown hyphae. As the root tissues are killed the foliage turns yellow, then brown and the whole tree dies. It is widely distributed on pines in tropical Africa including Nigeria (Gibson, 1979).

(xi) Little Leaf or Phytophthora Root Rot is caused by Phytophthora cinnamomi Rands. It is worldwide in distribution. This fungus is a soil-borne root pathogen. Long distance spread of P. cinnamomi is through contaminated soil or plants. Symptoms include a grey-green to brown top wilt of seedlings, a reddish colouration of the cambial region and rots of the feeding roots. The disease may affect nursery plants and young trees, but is usually associated with more mature trees (Ivory, 1987). Jehne (1971) recorded the disease on young Pinus radiata plantations in Australia.

(xii) Stem canker and die-back on pines caused by Botryodiplodia theobromae (Syn. Diplodia theobromae). The causal organism is a wound pathogen. Young P. elliottii and P. taeda have been reported killed by cankers of B. theobromae in Australia. The fungus is a common and important cause of blue stain in sawn timber in the tropics (Gibson, 1979).

2.5 Pathogenicity studies on conifers

Ivory (1975) inoculated spore suspensions of Amphichaetella echinata (Kleb.), a Fusarium sp., Glomerella cingulata and mycelial discs of Botryodplodia

theobromae, Cercospora pini-densiflorae and Lophodermium pinastri (Schrad. ex Fr.) Chev. on young shoots of Pinus caribaea and P. merkusii Jungh. and de Vriese. His inoculations did not produce any diseased condition after 2 months. Perhaps the "Inoculum - Host - Environment" in his experiments were not conducive to disease development.

Barrows - Broaddus (1981) inoculated two-year old slash (P. elliottii var. elliottii) and loblolly (P. taeda) pines with the fungus, F. moniliforme var. subglutinans Wollenw and Reink, the casual agent of pine pitch canker. Inoculations were made by needle puncture of the shoot epidermis through a droplet of conidial suspension. The fungus rapidly grew intercellularly from the cortex through the xylem rays to the pith. The parenchyma cell walls collapsed causing the cortex to disintegrate and produce gaps in the rays and pith. These observations were confirmed by Barrows-Broaddus and Dwinell (1983) in further inoculation studies.

Although F. moniliforme var. subglutinans generally attacks pines 17 years of age or older (Schmidt and Underhill, 1974), it has been reported to cause death of pine seedlings in nurseries (Barnard and Blakeslee, 1980). Barrows-Broaddus and Dwinell (1985) found the fungus to be

associated with stroboli, cones, and seed in slash and loblolly pine seed orchards in South Carolina. Pines vary in susceptibility to this pathogen. Dwinell (1978) inoculated 1 year-old seedlings and found shortleaf and Virginia pines highly susceptible; slash pine was intermediate in susceptibility and loblolly pine was most resistant to infection.

Inoculation studies with obligate parasites such as rusts on pines have also been recorded. The Comandra blister fungus (Cronartium comandrae Pk.) produced pycnia on 18 species or varieties of pines within 6 weeks to 8 months following exposure to infection (Mead et al., 1978). These authors worked with one to 6-months-old seedlings of 28 hard pine species. The seedlings were grown from seeds in the greenhouse and transferred in pots to the rust nursery during the time of natural release of basidiospores. When basidiospore dispersal had terminated, the seedlings were transferred to a greenhouse and observed in succeeding months for development of cankers, pycnia, and aecia.

Hood and Bell (1983) in Fiji, inoculated 98 potted Caribbean pine seedlings with woodblock cultures of two isolates of Ganoderma lucidum Sensu Lato. The inoculum

was from a basidiocarp on a dead tree of Pinus caribaea var. hondurensis. The inoculum remained active for 22 months in direct contact with seedling roots, but pathogenicity was not demonstrated.

Rees (1983), under controlled laboratory conditions, tested the pathogenic effect of some fungi obtained from pine seeds on 5-month-old sapplings. She observed wilting with Fusarium oxysporum, F. moniliforme and Botryodiplodia theobromae on Pinus caribaea and P. pocarpa.

The above studies confirm the fact that Fusarium spp., Diplodia sp., Botryodiplodia spp. are known pathogens of Pines. These pathogens could be carried in the soil, in/on seeds, plant parts or debris. They could enter their hosts through wounds or natural openings. They cause varying degrees of losses (stunted growth to death) at the seedling stage.

2.6 Control of seed and nursery diseases of conifers

(i) Chemical control. This has been in use over the years for the control of fungi on tree seeds and seedlings (Vaartaja, 1964; Harman and Nash, 1978; Bateman, 1983). Fatuga (1973) investigated the effect of Agrosan 5W (8.1% Totyl - mercury acetate), a seed-dressing fungicide on the

germination of the seeds of three species of Pine. The chemical was thoroughly mixed with weighed seed lots in closed polythene bags (0.075 gm to 100 gms seed). The seeds were then germinated on Petri dishes, in sterilized and unsterilized soil. The study showed that the chemical caused no significant increase in the emergence of seedlings. Ofong (1979) reported that seed pelleting with an othosticker and fungicides reduced germination of pine seeds whereas soil treatment with 0.01 g, 0.05 g, 0.1 g macuprax, thiram, difolatan and benlate dissolved in 100 ml tap water and 0.05, 0.25 and 0.5% dilution of tillex resulted in a high emergence of Pinus caribaea and P. oocarpa seedlings. Repeated application of the fungicides however increased seedling mortality.

Hong and Ivory (1974) investigated the feasibility of using mercuric chloride solution as a sterilant and its effect on germination after storage of seeds. They observed that many mercuric chloride treated seeds had the tips of their radicle blackened upon germination and noted that this might have undesirable effects on the continued development of the seedlings. Seeds sterilized in 0.1% mercuric chloride solution, air-dried (in an air-conditioned room), sealed in polythene packets and stored

for one month showed an increase in germination. One percent mercuric chloride treatment was not suitable.

Ivory (1975) found Benlate, Topsin M, Difolatan and Daconil effective in the control of "Brown needle disease" caused by Cercospora pini-densiflorae, Hori and Nambu. Singh et al. (1983) in a similar study on control of Cercospora needle blight of pines in nurseries obtained effective control by a spray of 0.6% dithane M-45 or cuman L on seedlings of P. roxburghii Sarg.

Palmer et al., (1986) found benomyl effective in controlling the shoot blight of pine caused by Sphaeropsis sapinea (Fr.) Dyko and Sutton (= Diplodia pinea (Desm.) Kickx). This fungicide is effective when applied twice at 14 days interval during the first year of growth of the seedlings.

Seed-borne infection is eradicated less readily by fungicidal treatments than expected from the response of the pathogen in vitro (Bateman, 1976). This could be due to antagonistic effect of seed-borne or soil-borne saprophytes or due to the location of the pathogen within the tissues of the seed or not. Bateman (1983) found the incomplete control of seed-borne F. nivale (Fr.) Ces. (by seed treatment with phenyl mercury acetate) to result from

pathogens located deep, within the seed and therefore inaccessibility ofthe pathogens to the fungicide.

(ii) Biological control. This has been effective on some fungal diseases of trees. Peniophora (Phlebia) gigantia (Fr.) Masse. has been used for the control of Heterobasidion annosus (Fr.) Bref. of pines in Britain (Rishbeth, 1963; Johnston and Booth, 1968). Trichoderma viride Pers ex. Fr. has also been successfully used in the control of Rhizoctonia solani (Johnston and Booth, 1968).

(iii) Other methods of control. Heating the seeds to a temperature lethal to fungi, but not to the seeds was found effective by Miller and McWhorter (1948) in the elimination of storage fungi like Phoma sp., Botrytis sp., Fusarium sp., Aspergillus sp. and Penicillium sp. Ivory (1987) recommended for the control of most diseases of pines: sowing of seeds at low density in an acid, well drained soil; watering sparingly and shading as little as possible; planting pines in un-infected areas; avoiding over-crowding in seed beds and nursery beds and using resistant varieties where available.

Eradication of damping-off by seed bed sterilization using heat before sowing has been widely used (Magnani, 1970). Magnani (1970) however noted that this method was

superior to other methods of control since it also controlled weeds, nematodes and led to the release of soil nutrients. Other fumigants for seed bed sterilization are formalin, methyl bromide and chloropicrin (Gibson, 1979).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sources of Seeds

Pinus caribaea var. *hondurensis* seeds from three seed lots were used for the investigation on seed-borne fungi. These included one seed lot with index number 7139 obtained from the Forestry Research Institute of Nigeria. This seed lot has been planted in Nigeria for the afforestation programme. It was originally obtained from the Forestry Commission, Alice Holt, U.K. The other two seed lots 12/81 and 50/78 were obtained directly from the Forestry Commission, Alice Holt, U.K. All the seeds were packed in cellophane bags and stored in the cold room at temperature of 5°C. Greaves (1978) provided the information on stand location of the seed lots (Appendix 2).

3.2 Culture media and sterilization of materials

(i) Agar culture media

All the agar culture media used were prepared by dissolving the appropriate quantity of the particular agar in sterile distilled water followed by autoclaving at 1.06 kg/sq. cm for 15 min. They were cooled to 45°C prior to pouring into 9 cm diameter sterile Petri-dishes. 15 ml

molten agar was poured per dish. The different agar media used were as follows:

Water Agar (WA)

10.0 gms Technical Agar No. 3

1 litre distilled water

Malt Extract Agar (MEA)

30.0 gms malt extract, Oxoid

20.0 gms Technical Agar No. 3

1 litre distilled water

Potato Dextrose Agar (PDA)

4.0 gms Potato Extract

20.0 gms Dextrose

15.0 gms Agar No. 1

1 litre distilled water.

(ii) Water and Glassware

Distilled water, glass Petri-dishes, pipettes, stirring rods, dispenser, filter papers were sterilized by autoclaving at 1.06 kg/sq. cm for 15 minutes. The glasswares were first wrapped in aluminium foil before autoclaving. Whenever sterile plastic Petri-dishes were available, they were used once and discarded.

(iii) Seeds

Whenever desired, seeds were surface sterilized by immersing them for 5 minutes in a solution of sodium hypochlorite containing 1% available chlorine. One percent of chlorine in the solution was obtained by diluting six fold a solution of sodium hypochlorite containing 6% chlorine with sterile distilled water.

(iv) Soil/Peat/Vermiculite rooting medium

This was sterilized by autoclaving at 1.06 kg/sq. cm. for 2 hours for 40 minutes.

3.3 Germination Tests

The usual "blotter" or rolled towel tests were used. Occasionally, the seed germination chamber (Copenhagen bath) in Forestry Research Institute, Ibadan, Nigeria was used. One hundred seeds replicated four times were placed on moist blotter and incubated at 27-30°C. Whenever necessary the seeds were surface sterilized, placed on 1% water agar in Petri-dishes and incubated at 20°C. The number of germinated seeds were recorded after 21 days.

Tetrazolium tests were done occasionally in order to determine the viability of the seeds. 1% tetrazolium solution was made by dissolving 20 g tetrazolium salt in a

800 cc buffer solution which was warmed to 60°C. The buffer solution consisted of 7.26 g KH_2PO_4 (Potassium dihydrogen orthophosphate) in 800cc distilled water. The tetrazolium solution was kept in the darkness at 30°C. To carry out the tetrazolium test, each seed was cut into two longitudinally such that the cut ran across the embryo of the seed. One half of the seed was discarded and the other half was soaked in distilled water for 16 hours. (Only one half was used because it was assumed that the two halves were symmetrical morphologically). The water was discarded and replaced with the tetrazolium solution for another 16 hours. The tetrazolium solution was then discarded and the seeds were put into a Petri-dish, covered with distilled water. The embryos were separated from the seeds by means of a mounted needle. The embryos were examined under the stereoscopic -microscope. Bright red staining of embryo showed viability.

3.4 Detection of Seed-borne fungi

The standard procedures used for the detection of seed-borne fungi were those described by the International Seed Testing Association (ISTA, 1976a&b) and Neergarrd (1977). These methods used for the determination of the health of seeds consisted of:

(i) direct inspection of dry seeds,

(ii) plating of seeds on blotter,

(iii) plating of seeds on agar,

(iv) seedling symptom test.

3.4.1 Direct inspection of dry seeds

The seeds were examined first with the naked eyes and later under the stereoscopic-microscope (at 40x) to establish their purity, to detect fungal structures (such as sclerotia), malformed and discoloured seeds and those which had holes from insect or mechanical damage. Four hundred seeds were examined from each seed lot. Each seed sample was separated into normal seeds, mechanically damaged seeds and seed-debris (broken cones and plant parts). Each group was then surface sterilized in 1% sodium hypochlorite for 5 minutes, plated on PDA, incubated at 22°C and examined for fungal infection.

3.4.2 Plating of seeds on blotter

Three layers of sterilized 9 cm - diameter Whatman No. 1 filter paper were soaked in sterile water and placed in 9 cm sterile plastic Petri-dishes. Twenty seeds surface sterilized for 5 mins in 1% sodium hypochlorite were placed in each Petri-dish. 100 seeds replicated four

times to give a total of 400 seeds were examined from each seed lot. The Petri-dishes were incubated for 7 days under alternating cycle of 12 hours near ultra violet (N.U.V.) light and 12 hours darkness and a temperature of 20°C ± 2. This incubation condition was supplied by black light fluorescent lamps which emitted light mainly at wavelength near 3,650 (365 nm), and cool white daylight fluorescent lamps which emitted some NUV light. NUV stimulates sporulation in many seed-borne fungi (Neergaard, 1977). In each seed lot too, some seeds which were not surface sterilized were plated on blotter.

3.4.3 Plating of seeds on agar

The seeds were surface sterilized by soaking for 5 minutes in 1% sodium hypochlorite. Excess solution was drained off. Ten seeds were placed in each Petri-dish containing Potato Dextrose Agar. Forty plates were used for each seed lot. 100 seeds replicated four times to give a total of 400 seeds were examined from each of the three seed lots. The Petri-dishes were incubated as in the blotter method.

3.4.4 Seedling-symptom test

The aim of this test was to find out if symptoms of disease could be detected in seedlings. One thousand two hundred seeds from batch 7139 observed in the preliminary investigation to habour a high percentage of pathogens were used. The seeds were wrapped in muslin cloth and soaked in tap water for 48 hours. The seeds were then surface sterilized in 1% sodium hypochlorite solution for 5 minutes and then sown in a sterilized sowing medium in wooden boxes. The sowing medium consisted of a mixture of sieved coco-nut fibres and crushed granite (ratio 1:1 v/v). The sowing medium was sterilized in a soil-sterilizer at 150°C for 1 hour. Four seedling boxes (61 x 46 x 7) cm^3 were filled with the sterile sowing medium up to 6 cm deep and wetted with water. Each box was divided into 4 compartments. 300 holes were made in each compartment and one seed was dropped in each hole and covered lightly with the sterile sowing medium. For the control, one thousand two hundred seeds which were not surface sterilized were planted in the sterilized sowing medium. The sowing medium was moistened twice daily with ordinary tap water. The seed boxes were covered with thick white polythene sheets to

maintain a high humidity, favourable for seed germination. The seed boxes were kept in the Greenhouse of Forestry Research Institute of Nigeria, Ibadan, with a relative humidity of 65% ± 5 and temperature of 27°C ± 2. Plants were thus kept from outside infection. From the 5th-30th day of sowing, the germinated seedlings were counted, picked, inoculated with mycorrhizal fungus, which is necessary for pine survival in Nigeria, Ekwebelam and Odeyinde (1983) and transplanted into black polythene bags. (The germination percentage of the seeds was recorded on the 30th day). The potting mixture was that used by Okorie and Njoku (1979). It consisted of sterile fine sand and top soil (1:1 v/v) plus 2.5 kg superphosphate/m^3 of soil. One thousand black polythene pots (540 cm^3 capacity) were filled with the sterile potting mixture and wetted with water. A hole was dibbled in the centre of the pot. The mycorrhizal fungus used for inoculation was Pisolithus tinctorius (Pers.) Coker and Cooch. This fungus was stored on malt extract agar (MEA) at the Forestry Research Institute, Ibadan. The fungus was subcultured on Petri plates containing MEA and incubated at 27°C. The inoculum suspension was made by scrapping 14 day old culture into sterile distilled water

using a sterile scalpel (50 ml/Petri dish). The suspension was poured into McCartney bottles. The radicle of seedling to be inoculated was dipped into the suspension and the radicle propped in the potting mixture. One seedling was transferred into each pot. The seedlings were kept in the Greenhouse for 24 hours. They were later moved to the open and watered twice daily. The seedlings in the polythene pots were observed for mortality and symptoms of disease for 6 months.

3.4.5 Isolation and identification of fungi

Incubated seeds were observed under a low power stereocopic-microscope with up to 40x magnification. A stereoscopic-microscope enabled observation of the fungi on their host in situ, undisturbed in a condition of natural growth. Light from the stereo-microscope was shone from above to the plates. Lamps were placed at 60°. Seeds were individually examined for seed-borne fungi. For each fungal species, using the compound microscope at 400x, notes were taken of characteristic features such as form, length and arrangement of conidiophores; the form, size, septation, colour, chain formation of conidia and their arrangement on the conidiophores; appearance of

spore masses; characters of mycelium; and density of colour of mycelia.

Visible colonies were removed and subcultured. This was done by picking part of mycelium or conidia from the seeds with a flame-sterilized needle and transferring to MEA/PDA. When in pure culture, detailed identification of isolate was attempted. This involved classification into genera. Where a name could not be given to an isolate, an isolate-code was used. Fungal tissue was stained in cotton blue and lactophenol and observed under the compound microscope (Wild, Hearbugg, Switzerland and Olympus, Japan). Reference slides of each species were made semi-permanent with Glycerol, made by Gurr. Fungal isolates were identified with the aid of the following texts: Barnett and Hunter (1972), Booth (1971, 1977), Ellis (1971, 1976) and Hawksworth et al. (1976). Even with the aid of these books, most fungi could only be identified to genera. For identification to species level, isolates were sent to the Commonwealth Mycological Institute, Kew, Surrey.

3.5 Pathogenicity Tests

Four fungal species which are known to be pathogenic and which were observed most frequently from pine seeds during previous experiments were tested for their pathogenic effects on seeds and seedling of 7139 Pinus caribaea var. hondurensis. The fungal species tested were Fusarium moniliforme var. intermedium Neish & Leggett, Fusarium equiseti (corda) Sacc., Phialophora fastigiata (Lagerb & Melin) Conant and Aspergillus niger van Tiegnum.

3.5.1 Pathogenicity on seeds

Each fungus was subcultured on plates of PDA and incubated for 5 days at 22°C. 200 seeds were used for each fungus. All the seeds were surface sterilized for 5 minutes in 1% sodium hypochlorite solution. The seeds were divided into two batches. One batch was put on the culture in the plate. The plate was turned by hand six times in clockwise and anticlockwise directions. The aim here was to cover the seed surface with the inoculum. The seeds remained in the plates overnight. The other batch, which served as control, was rolled around on PDA without the fungus. Twenty seeds were plated on PDA, incubated at 22°C for 21 days. At the end of the incubation period, seed germination and disease development were assessed.

3.5.2 Pathogenicity on seedlings

A Raising of seedlings

(i) 3-week-old seedlings

These were raised from seeds which were surface sterilized in 1% sodium hypochlorite for 5 minutes. The seeds were plated in 1% WA and incubated for 3 weeks.

(ii) Three-month-old seedlings

These were obtained by aseptically transferring three-week-old seedlings from WA plates into Vermiculite and peat mixture in Petri-dishes. The rooting mixture was obtained by adding vermiculite plus peat (4:1 v/v) plus 500 ml distilled water plus 9.22 g phostrogen. The mixture was sterilized in the autoclave at 121°C for 40 minutes. In order to transfer a seedling into the rooting medium, one side of an empty, sterile plastic Petri dish was cut with a red-hot scalpel. The dish was filled with peat-vermiculite mixture, the three-month old seedling was then placed into the dish and the cut end of the dish was sealed with solid petroleum jelly ("Vaseline" Cheesebrough Ponds Ltd., London) to ensure that there was no contamination of the rooting mixture from the air. The dish was also sealed with P.V.C. electrical insulation tape to reduce evaporation of moisture from the dish.

Finally, the dish was wrapped with aluminium foil to create a dark, near-soil condition for the growth of the root. These three-month old seedlings were kept in a high humidity chamber (provided by a seed tray plus its cover) Plate 1. This was closed for three days. The vents of the chamber were opened in the next 4-6 days and by the 7th day the cover of the chamber was taken off. This treatment "hardened" the seedlings. It enabled them to make a gradual adjustment from their initial agar plate environment to the new laboratory atmosphere. The treatment ensured high survival of seedlings.

B Inoculum preparation

Inoculum used consisted of:

(i) 5 mm mycelia plugs of each of the fungal species obtained from 5-day-old cultures on PDA plates.

(ii) Spore suspension obtained by washing the spores of 5-day-old cultures of Aspergillus niger in sterile distilled water. A drop of "Tween" 80 was added to disperse the spores uniformly in the suspension.

(iii) Spore suspensions of Fusarium moniliforme var. intermedium, F. equiseti and Phialophora fastigiata were prepared by the method of Cappellini and Peterson (1965).

Plate 1. The inoculated seedlings of Pinus caribaea var. hondurensis in the "humidity chamber".

This method was used to obtain large numbers of spores in culture. The medium known as carboxymethylcellulose (CMC) liquid medium contained:

NH_4NO_3	-	1 g
KH_2PO_4	-	1 g
$MgSO_4.7H_2O$	-	0.5 g
Yeast extract	-	1.0 g
CMC	-	15.0 g
H_2O	-	1 litre

40 ml of the medium was dispensed into 250 ml Erlenmeyer flask. The medium was autoclaved at 120°C for 15 min. One Petri plate culture was scraped with a sterilized scalpel, macerated with the scalpel and the mycelial pieces were added unto the CMC medium. The flasks were incubated at 25°C and shaken on a rotary shaker at 100 rpm. for 4 days. The spores produced were centrifuged at 1800 rpm for 5 min. and resuspended in 10 ml sterile distilled water. The number of spores/ml was measured with the haemocytometer and adjusted to 7×10^7.

C Inoculation techniques

Ten seedlings were inoculated with each fungus. Five seedlings served as control.

(i) Radicle inoculation

A 5 mm inoculum-agar plug was placed at the junction of radicle and plumule of 3-week-old seedlings in 1% WA plates. The control seedlings received ordinary PDA plugs without fungi.

(ii) Stem-wound inoculation

Three month old seedlings were used. The stem point to be inoculated was usually 2 cm above the soil level. This area was surface sterilized by swabbing with cotton-wool soaked in 95% alcohol. A puncture (1 mm deep) was made into the stem with an alcohol and flame sterilized needle. The spore suspension, or sterile distilled water in the case of the controls, was injected into the wound with a disposable sterile needle and syringe to point of run-off. Cut surfaces were sealed with solid petroleum jelly.

(iii) Root inoculation

5 pieces of 5 mm. inoculum agar plugs were placed on the roots of 3-month old seedling in the peat-vermiculite rooting mixture.

(iv) Shoot-Spray inoculation

Two ml spore suspension was sprayed on the aerial part of each 3-month old seedling. These seedlings were

kept in the high humidity chamber (already described under raising of seedlings). A high humidity was maintained in the chamber by spraying the enclosure with water and by the help of cotton-wool saturated with water.

3.5.3 Sectioning technique

Seedling health was observed at regular weekly intervals for one month. Stem and root sections were taken from control and 3-month old seedlings which were inoculated through stem-wound. The sections were fixed in acrolein (Feder and O'Brien, 1968) and embedded in polyethylene glycol (PEG). The tissues were cut into 1 cm pieces and treated as follows:

10% aqueous acrolein (vacuum infiltration)	3 mins
10% aqueous acrolein	12 hrs soak.
H_2O (3 changes)	12 hrs
15% polyethylene glycol soln.	12 hrs
25% polyethylene glycol soln.	12 hrs
50% polyethylene glycol soln.	12 hrs
75% polyethylene glycol soln.	12 hrs
100% polyethylene glycol soln.	12 hrs

Tissues were finally embedded in polyethylene glycol (3 part polyethelene 4000 + 1 part polyethylene glycol

1,500 + 2% glycerol). Sections (20μ) were cut with the sliding microtome and stained in aniline blue (WS) in lactophenol.

3.6 Effect of different storage temperatures on invasion of seeds of Pinus caribaea var. hondurensis by some seed-borne fungi

The invasion of four fungal species on seeds of Pinus caribaea var. hondurensis, index no. 7139, under three temperature regimes (5°, 20° and 30°C) was studied. The fungi examined were F. moniliforme var. intermedium, F. equiseti, Phialophora fastigiata and A. niger.

Each fungus was subcultured on 3 plates of PDA and incubated for 5 days. 3,000 seeds were counted out, divided into 3 batches and each batch rolled around on the surface of the culture and left on the fungus overnight (see section 3.5.1). Each Petri-plate content was put into a McCartney bottle, sealed and stored at 5°C, 20°C and 30°C. At 24 hours, 2 and 8 weeks, 200 seeds were taken out from each treatment. These were surface sterilized for 5 minutes in 1% sodium hypochlorite and 100 were plated on PDA, incubated for seven days as for the blotter technique (see 3.4.2) to observe the presence of

fungi. The remaining 100 seeds were plated on 1% Water Agar (WA) and germination was assessed at 21 days. The health of emergent seedlings was also noted. For the control, the seeds were rolled over PDA plates without the fungus.

3.7 Location of fungi on Pinus caribaea seeds through component plating of seed parts

The aim here was to be able to provide information on the presence and location of any internally seed-borne fungi of Pinus caribaea seeds.

100 seeds from 7139 were used in this study. The seeds were surface sterilized for 5 minutes in 1% sodium hypochlorite, rinsed in sterile water and then soaked at the rate of one seed per McCartney bottle in 5 ml sterile distilled water. The seeds were soaked for 16 hours. Each seed was then dissected out into component parts; the seedcoat, the endosperm and the embryo. These three main seed components were placed in numbered positions on PDA in three separate plates at a rate of 5 seed pieces per plate. The plates were incubated for 7 days.

Fungi developing on the seed pieces were isolated, identified and recorded as previously detailed in Section 3.4.5

3.8 Location of fungi on Pinus caribaea seeds by means of scanning electron microscopy (SEM)

In this study SEM was used to investigate the structure of the seed coat surface, to study the location of fungal spores and hyphal fragments on the seed surface and to study the attachment of hyphal structures to the emerging radicle.

Materials examined consisted of:

(a) Dry seeds which had been stored at 5°C. These were examined to study the structure of the seed surface and to study the location of spores or fungal structures on the seed coat.

(b) Seeds, surface sterilized for 5 minutes in 1% sodium hypochlorite, incubated by the blotter technique for seven days and observed to be naturally covered with mycelia and spores of Aspergillus niger.

(c) Seeds, as in (b) but covered with Phialophora fastigiata.

(d) Pure culture of Aspergillus niger on PDA.

(e) Pure culture of Phialophora fastigiata.

Specimens (b) to (e) were prepared in order to examine spores and mycelia of the named fungi with the SEM. Such observation aided the identification of similar fungi on the dry seeds examined in (a) above.

(f) Seeds, artificially inoculated with Aspergillus niger spores (see 3.5.1 on pathogenicity on seeds) and incubated by the blotter technique for 0, 12, 24 and 36 hours. This was to study the penetration of germinating spores into the seed coat and the radicle.

Material (a) which consisted of ordinary dry seed was mounted on aluminium stub with a quick set epoxy adhesive (R.S. Components Ltd. Northents, U.K.). After mounting, the specimen was coated with a 30 µm thick layer of gold in a Polaron E 500 Sputter Coater and examined in a Cambridge Stereoscan 150 at an accelerated voltage of 5 KV.

The other specimens (b) to (f) which consisted of seeds covered with spores and mycelia and pure cultures

were held in covered specimen bottles and fixed as follows:

1% Osmium tetroxide (fixation)	overnight soak 12 hours
0.1M sodium cacodylate	3 X 10 minutes rinses
50% ethanol	10 minutes soak
70% ethanol	10 minutes soak
90% ethanol	10 minutes soak
95% ethanol	10 minutes soak
100% ethanol	2 X 10 minutes rinses
Ethanol/Acetone 2:1	10 minutes soak
Ethanol/Acetone 1:1	10 minutes soak
Ethanol/Acetone 1:2	10 minutes soak
Absolute acetone	overnight soak

The specimens were then removed from acetone, critical point dried, mounted and examined in the same way as dry seed.

3.9 In vitro studies of the fungicidal action of three plant protection chemicals against certain seed-borne fungi of Pinus caribaea

Three plant protection chemicals: Benlate, Thiram and Captan were evaluated in vitro to ascertain their effectiveness in the control of seed-borne fungi found in

this study to be prominent on Pinus caribaea seeds. Benlate is known as Benomyl or methyl 1-(butylcarbomyl)-2-benzimidazole carbamate or 50% methyl N-Benzimi-dazole-2-yl-N (butyl carbomoyl carbamate). Thiram is Fernasan or TMTD (Tetramethyl thiuram disulphide) and Captan is 2-dicarboximide or N-trichloromethyl-thio-4-cyclohexene-1. These chemicals were obtained from the Forest Pathology Unit of the Oxford Forestry Institute, Oxford, U.K. The fungi used were Fusarium moniliforme var. intermedium, Aspergillus niger and Phialophora fastigiata.

A 4000 ppm suspension of active ingredient of each chemical was prepared in sterile distilled water. A dilution series was made from this to give concentrations of 2000, 1000, 200, 100, 40, 20 and 10 ppm. Each concentration was added unto hot-molten PDA (ratio 1:9 v/v) in bottles, shaken thoroughly and dispensed into 5 cm Petri-dishes to give a concentration of 400, 200, 100, 40, 20, 10, 4, 2 and 1 ppm active ingredient. Each dish had 8 ml agar-fungicide added using a sterilized dispenser. Plates of PDA not supplemented with the chemicals served as controls. The bottom of each Petri-dish was marked with two perpendicular lines passing through the centre. When the agar had solidified, 5 mm agar disc of each

fungus was placed in the centre of the plate, with the aid of a flame-sterilized 5 mm agar disc borer. Four replicates of each fungicide concentration were used. Inoculum was obtained from 5 day-old cultures of Fusarium moniliforme var. intermedium, Aspergillus niger and 8 day-old cultures of the Phialophora fastigiata. Each fungus was grown on three PDA plates. The culture from one plate served as inoculum for each fungicide. The Petri-dishes were randomly arranged in the incubator. They were incubated at 22°C. For A. niger, the 5 mm agar-well had 0.2 ml spore suspension.

The linear growth of the fungus was measured along two perpendicular radii for each plate. Readings were taken at 1- or 2-day intervals until the whole plate was almost covered in the control plate. Eight measurements were recorded for each treatment. The mean of the eight measurements was recorded as the growth of the fungus in each treatment. The means were analysed using analysis of variance and Duncan's multiple range test (Little and Hills, 1972).

CHAPTER FOUR

R E S U L T S

4.4 Detection of seed-borne fungi on Pinus caribaea

4.4.1 Direct inspection of dry seeds

Examination of seeds showed that 83.6% appeared normal, 11.0% were damaged mechanically or by insects and had cracks or holes and 5.4% were debris made up of broken pieces of cones, twigs, testa and stones (Plate 2). P. caribaea index no. 7139 had the highest percentage of normal seeds (95.1%) and the least percentage of debris (Table 1).

The mechanically damaged seeds contained mainly Aspergillus flavus, Aspergillus niger, Fusarium moniliforme var. intermedium and Phomopsis occulta (Table 2).

The seed debris consisted of 60% cones, 30% stones and 10% plant parts. The debris had a large population of mycoflora. These consisted mainly of Aspergillus niger, Chaetomium globosum, Fusarium equiseti, Fusarium moniliforme var. intermedium, Macrophoma sp., Pestalotiopsis sp. and Phialophora fastigiata (Table 3).

Plate 2: Collection of seeds and plant-debris of Pinus caribaea var. hondurensis
e = cone piece
n = normal seed
s = insect/mechanically damaged seed.

Table 1. Percentage occurrence of normal seeds, mechanically damaged seeds and debris in some *Pinus caribaea* var. *hondurensis*.

Index No.	Percentage		
	Normal seeds	Mechanically damaged	Debris
7139	95.1	3.6	1.3
50/78	75.4	15.0	9.6
12/81	80.2	14.4	5.4

Table 2. Percentage infection by fungi of mechanically damaged Pinus caribaea var. hondurensis seeds (after pretreatment in 1% NaOCl for 5 minutes) on PDA.

Fungi identified	Percentage infection*		
	7139	50/78	12/81
Aspergillus flavus	38.0	27.0	30.0
A. niger	21.0	30.0	39.0
Fusarium moniliforme var. intermedium	20.0	24.0	32.0
Phomopsis occulta	11.0	16.0	12.0

*multiple infection ∴ total > 100.

Table 3. Percentage infection of Pinus caribaea var. hondurensis seed debris (after pretreatment in 1% NaOCl for 5 minutes) on PDA.

Fungi identified	Percentage infection*		
	7139	50/78	12/81
Aspergillus niger	20.0	15.0	17.5
Chaetomium globosum	20.0	15.0	25.0
Fusarium equiseti	5.0	12.5	0.0
F. moniliforme var. intermedium	12.5	20.0	32.5
Macrophoma spp.	0.0	35.0	25.0
Pestalotiopsis spp.	0.0	30.0	20.0
Phialophora fastigiata	5.0	17.5	12.5

*multiple infection ∴ total > 100.

4.4.2 Detection of fungi on blotter

When seeds which were not pretreated by surface sterilization in 1% sodium hypochlorite were plated out on blotter, or agar, fast growing saprophytes, like Aspergillus sp. and Rhizopus sp. covered the whole dish and masked the observation of other fungi. Pretreating the seeds allowed for the expression of some seed-borne fungi such as Fusarium sp., Botryodiplodia theobromae and Cladosporium oxysporum. Therefore, only records on surface sterilized seeds are presented. The following fungi were observed: Acromonium sp., Aspergillus flavus, A. fumigatus, A. niger, A. tamarii, Botrytis sp., Botryodiplodia theobromae, Chaetomium funicola, Ch. globosum, Ch. indicum, Cladosporium oxysporum, Dendrophoma sp., Fusarium equiseti, F. moniliforme var. intermedium, Gliocladium roseum, Macrophoma spp., Mucor spp., Paecilomyces variotii, Penicillium citrinum, P. cyclopium, Pestalotiopsis spp., Phialophora fastigiata, Phomopsis occulta., Rhizopus sp. and Trichoderma sp. The percentage occurrence of these fungi are shown in Table 4. The mean percentage of occurrence ranged from 0.5% to 72.0%.

Table 4. Percentage* infection of Pinus caribaea var. hondurensis seeds+ on blotter.

Fungi identified**	7139	50/78	12/81
1. Acromonium sp.	5.8	0.0	0.0
2. Aspergillus flavus	10.0	14.5	8.0
3. A. fumigatus	8.0	12.3	14.5
4. A. niger	72.0	32.0	18.5
5. A. tamarii	4.5	0.0	0.0
6. Botryodiplodia theobromae	0.0	0.0	5.8
7. Botrytis sp.	0.0	0.0	0.5
8. Chaetomium funicola	2.0	10.0	5.3
9. C. globosum	15.3	25.0	18.5
10. C. indicum	4.5	0.0	0.0
11. Cladosporium oxysporum	0.0	4.0	8.8
12. Dendrophoma sp.	0.0	2.3	4.3
13. Fusarium equiseti	13.8	18.5	16.8
14. F. moniliforme var. intermedium	24.5	28.3	14.5
15. Gliocladium roseum	14.0	20.5	12.5
16. Macrophoma sp.	0.0	25.3	18.8
17. Mucor sp.	19.5	0.0	5.5
18. Paecilomyces variotii	0.0	5.0	8.0
19. Penicillium citrinum	0.0	14.5	10.5
20. P. cyclopium	0.0	2.5	4.5
21. Pestalotiopsis sp.	0.0	24.5	13.8
22. Phialophora fastigiata	22.3	0.0	18.5
23. Phomopsis occulta	0.0	8.3	12.5
24. Rhizopus sp.	18.8	11.5	5.5
25. Trichoderma sp.	0.0	0.0	6.0

* mean of 4 replicates

** multiple infection of seeds ∴ total > 100.

\+ One hundred seeds examined

4.4.3 Detection of fungi on agar

The fungi identified using the agar test on seeds which were surface sterilized for 5 minutes in 1% sodium hypochlorite were: Aspergillus flavus, A. niger, Botryodiplodia theobromae, Cladosporium oxysporum, Fusarium equiseti, F. moniliforme var. intermedium, Macrophoma sp., Penicillium citrinum, Pestalotiopsis spp., Phialophora fastigiata and Phomopsis occulta. The mean percentage range of occurrence was 2.5% to 68.5% (Table 5).

4.4.4 Seedling symptom test

The seeds which were surface sterilized for 5 minutes in 1% sodium hypochlorite solution and planted in sterilized sowing medium gave a germination percentage of 42, while the control gave 20 (Table 6).

In the surface sterilized seeds sown in sterilized sowing medium, 10% of the emerging seedlings showed wilting and "water-soaked" appearance and died between the 5-30th day of sowing. This same disease symptom was observed in 12% of the control.

Table 5. Percentage* fungal infection of 100 Pinus caribaea var. hondurensis seeds on PDA.

Fungi identified**	Percentage infection		
	7139	50/78	12/81
Aspergillus flavus	13.3	9.5	6.0
A. niger	68.5	32.8	20.0
Botryodiplodia theobromae	0.0	0.0	4.0
Cladosporium oxysporum	0.0	0.0	2.5
Fusarium equiseti	18.3	16.5	24.3
F. moniliforme var. intermedium	30.0	34.3	15.8
Macrophoma sp.	0.0	32.8	34.5
Penicillium citrinum	0.0	12.3	18.5
Pestalotiopsis sp.	0.0	32.5	19.0
Phialophora fastigiata	9.8	11.8	12.5
Phomopsis occulta	8.8	5.5	0.0

* Mean of 4 replicates, each of 100 seeds.

** The percentage for individual fungi are the total of isolates in which the fungus was recovered alone or in combination with another fungus. For this reason the totals exceed 100% (multiple infection).

Table 6. Germination and survival of seeds of *Pinus caribaea* var. *hondurensis* in sterilized sowing/potting mixture.

	Sterilized seeds					Unsterilized seeds				
Replicates	1	2	3	4	Mean	1	2	3	4	Mean
% germination (30 days)	38	46	40	44	42	24	22	19	15	20
% seedling survival (1 month)	85	90	90	95	90	90	92	90	80	88
% mortality (1 month)	15	10	10	5	10	10	8	10	20	12
% mortality (6 months)	2	4	6	8	5	5	8	4	7	6

At the end of the experiment (6 months), mortality due to damping-off and wilting was observed in 5% of the seedlings from surface sterilized seeds planted in sterile soil and 6% in the control. The disease symptoms observed throughout the period of study were damping-off (that is, water-soaked appearance and collapse of the seedlings at the soil level), wilting and seedling blight. Culture and slides prepared from the dead seedlings showed the presence of F. moniliforme var. intermedium, Botryodiplodia theobromae, Rhizoctonia spp. and Rhizopus spp.

4.5 Pathogenicity Studies

4.5.1 On seeds

Aspergillus niger: This fungus resulted in a lower germination of seeds (18%) compared with the control which was 48%. It also delayed germination by three days. The emerging seedings were stunted in growth (Plate 3), the radicles turned brown or necrotic.

Fusarium equiseti: The seeds inoculated by this fungus gave a lower germination percentage of 23 compared with the control which was 50. Germination was delayed by

six days. The emerging seedlings were stunted in growth and their radicles turned brown.

Fusarium moniliforme var. intermedium: This fungus reduced the seed germination to 25% from 48 for the control. The emergent of seedling was not delayed but the seedlings were reduced in growth.

Phialophora fastigiata: This fungus had no effect on the germination of the seeds or the time of emergence of the seedlings.

Table 7 is a summary of the effect of the various fungi on the seeds and seedlings of Pinus caribaea var. hondurensis.

Plate 3: Stunted growth observed 3 weeks after inoculation of Pinus caribaea var. hondurensis seeds with Aspergillus niger.
a = normal growth (control)
b = stunted growth (inoculated)

Table 7. Effect of some seed-borne fungi on the germination of Pinus caribaea var. hondurensis

Inoculum	Inoculated seeds		Control	
	Germination (%)	Radicle appearance (days)	Germination (%)	Radicle appearance (days)
A. niger	18	10	48	7
F. moniliforme var. intermedium	25	8	48	8
F. equiseti	23	13	49	7
Phialophora fastigiata	48	7	47	7

4.5.2 Inoculated seedlings

Aspergillus niger inoculation: Inoculated seedlings appeared not to be affected by this fungus. The seedlings grew normally and no mortality was recorded. The fungus was not re-isolated from inoculated seedlings.

Fusarium equiseti inoculation: Pale pink floccose mycelia were produced in areas inoculated (the radicle, the roots, stems and leaves). The affected areas wilted and the plant died within 2 weeks of inoculation. The seedling mortality ranged from 30-40%. The fungus was re-isolated from all inoculated seedlings.

Fusarium moniliforme var. *intermedium* inoculation: The fungus grew and sporulated profusely in all inoculated areas of the radicle, roots, stems and needles (Plates 4-7). It caused the death of seedlings within 7 days of inoculation. The mycelium was observed and recovered from the needles, stems, roots and the radicle. Such mycelia-covered areas eventually wilted. The infected roots turned brown (necrotic) and the seedlings died. The seedling mortality was 20-60%.

Phialophora fastigiata inoculation: This fungus appeared to have no effect on the inoculated seedlings. Seedling growth appeared not be disturbed by the fungus. The fungus was not re-isolated from inoculated seedlings.

The summary of the result of the inoculation study is presented in Table 8.

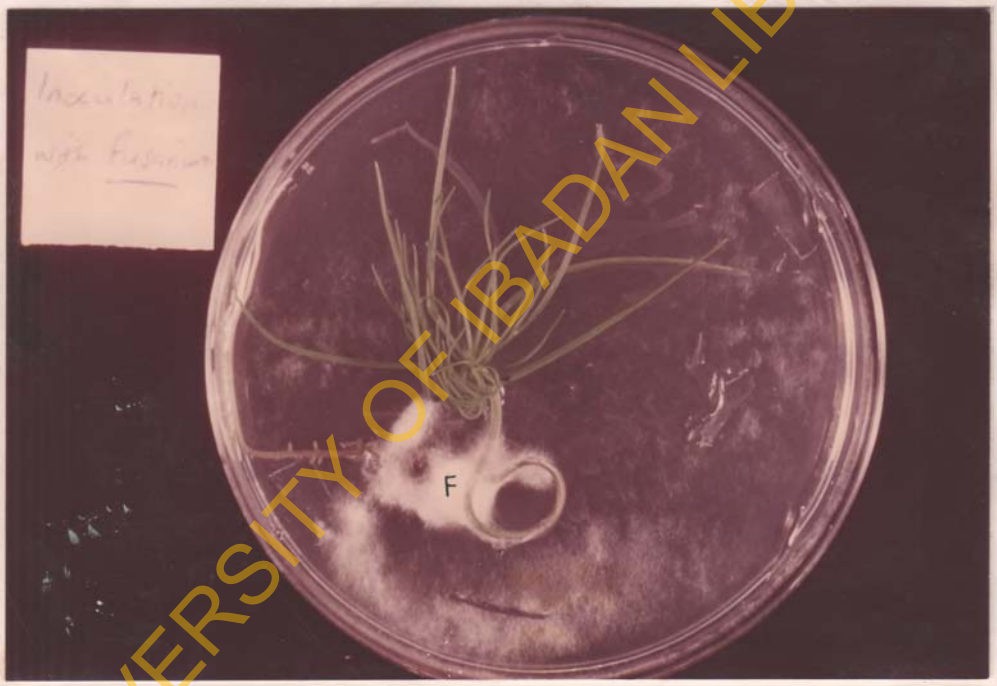

Plate 4: Three week-old seedling of Pinus caribaea var. hondurensis in PDA plate inoculated by placing mycelial discs of Fusarium moniliforme var. intermedium on the radicle.
Note the profuse growth of the fungus (F).

Plate 5: Three month-old seedlings of Pinus caribaea var. hondurensis inoculated by placing mycelial discs of Fusarium moniliforme var. intermedium on the root.
i = inoculated seedling showing wilt.
c = control seedling

Plate 6: Three month-old seedling of Pinus caribaea var. hondurensis inoculated by wounding the stem and injecting spore suspension of Fusarium moniliforme var. intermedium. Note the wilting of some needles.

C W

Plate 7: Three month-old seedling of Pinus caribaea var. hondurensis inoculated by aerial spray of spore suspension of Fusarium moniliforme var. intermedium.
c = control seedling
w = inoculated seedling showing profuse mycelia of the fungus and death of seedling.

Table 8. Effect of some seed-borne fungi on inoculated 3 month-old Pinus caribaea var. hondurensis seedlings

Inoculated seedlings			No. with disease symptom			CONTROL	
Fungus/inoculum	Inoculation technique	No. of plants inoculated	Wilt	Necrotic growth	Mycelial growth	No plants inoculated	Disease symptom
Fusarium moniliforme var. intermedium	Aerial spray	10	2	2	10	5	0
	Shoot-wound	10	6	10	10	5	0
	Mycelia disc root	10	3	3	10	5	0
Fusarium equiseti	Aerial spray	10	3	3	10	5	0
	Shoot-wound	10	4	4	10	5	0
	Mycelia disc root	10	4	4	10	5	0
Phialophora fastigiata	Aerial spral	10	0	0	0	5	0
	Shoot-wound	10	0	0	0	5	0
	Mycelia disc root	10	0	0	0	5	0
Aspergillus niger	Aerial spray	10	0	0	0	5	0
	Shoot-wound	10	0	0	0	5	0
	Mycelia disc root	10	0	0	0	5	0

4.5.3 Sectioning

The transverse section through the internode of a 3-month old seedling of Pinus caribaea var. hondurensis shows the epidermis, followed by the phloem and the cortex which is typically parenchymatous (Esau, 1965), Plate 8. A transverse section through an internode which has been artificially infected for 3 months showed that the whole of the surface appeared water-soaked to the naked eyes. When such transverse section was stained with aniline blue in lactophenol and examined under the compound microscope at 40x and 400x, the parenchyma cells and fibres were disintegrated and fungi hyphae penetrated and blocked the tracheids resulting in wilt (Plate 9).

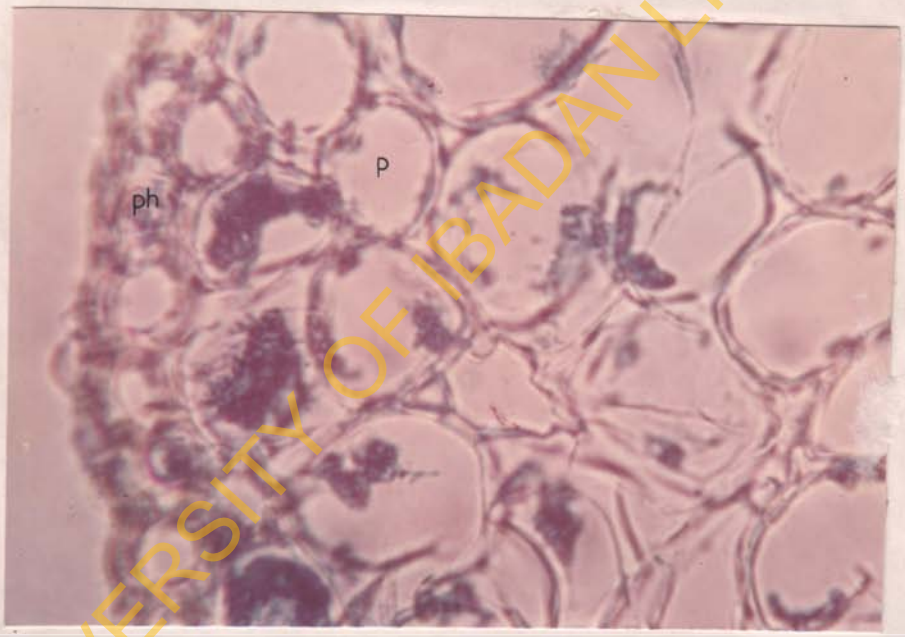

Plate 8: Transverse section through healthy stem of 3 month-old Pinus caribaea var. hondurensis showing the intact parenchyma (p) and the phloem (ph). x 400.

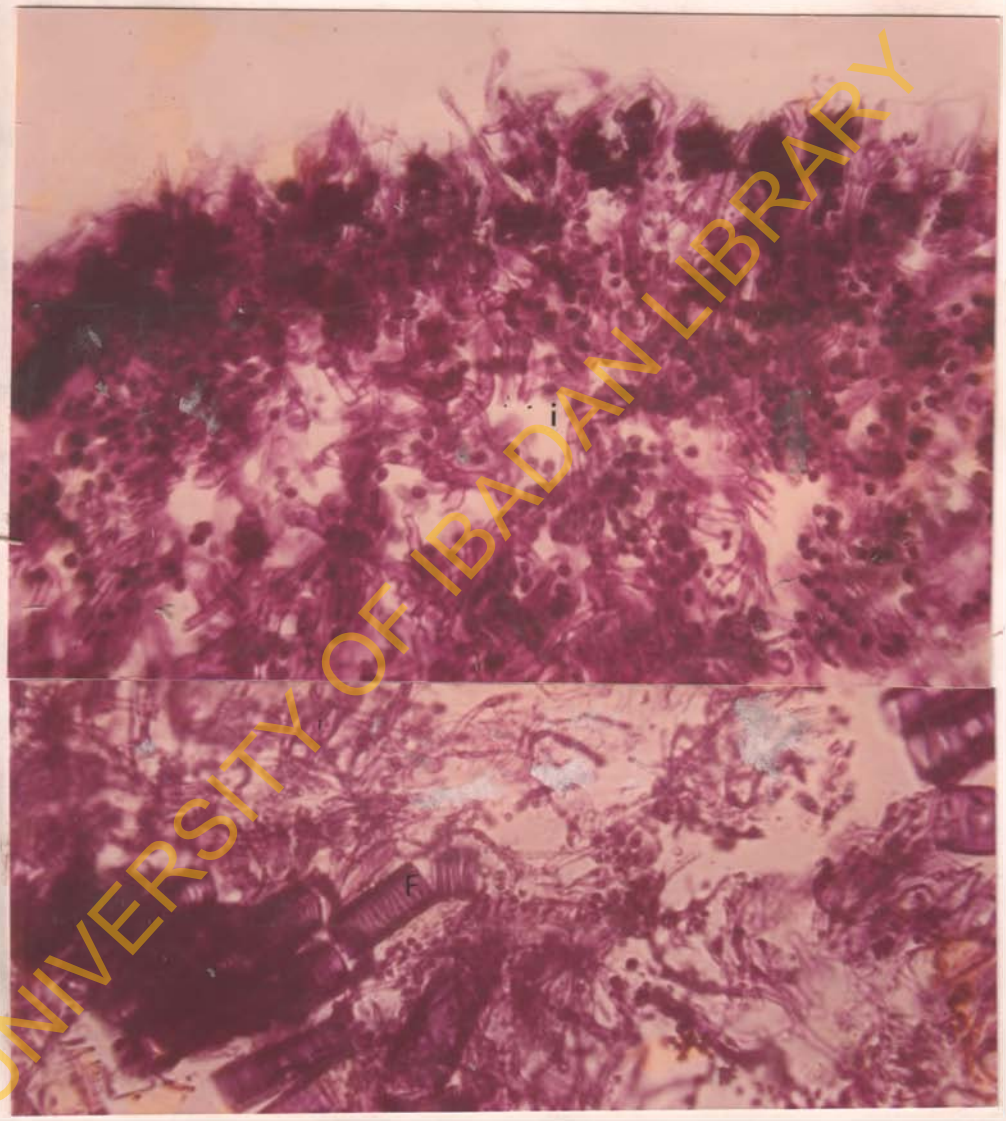

Plate 9: Transverse section through the stem of 3 month-old Pinus caribaea var. hondurensis inoculated with Fusarium moniliforme var. intermedium showing disintegrated fibres (F) parenchyma and fungal structures (i). x 400.

4.6 Effect of different storage temperatures on invasion of seeds of Pinus caribaea var. hondurensis by some seed-borne fungi

The recoveries of Aspergillus niger, Fusarium equiseti and Fusarium moniliforme var. intermedium were high throughout the eight weeks in the inoculated seeds (58-84%). Whereas the recovery of Phialophora fastigiata from inoculated seeds was 0-8% at the end of the eight weeks study. The recovery of Phialophora fastigiata was low when the other fungi were high perhaps because this fungus is a weak saprophyte.

In both inoculated and uninoculated seeds which were stored at 20° and 30°C, the percentage germination of the seeds dropped drastically from a range of 42-46% to 0%. But the percentage germination of those seeds that were stored at 5°C dropped to 17%. After eight weeks of storage at 5°C, the control seeds gave a 40% germination; seeds inoculated with Phialophora fastigiata gave 35%; those inoculated with Fusarium equiseti gave 20%; those with Fusarium moniliforme var. intermedium 18% and those with Aspergillus niger 17% (Table 9). Seedlings which

Table 9. Effect of storage temperature on percentage fungal isolate of inoculated Pinus caribaea var. hondurensis seeds.

*Inoculum	Storage Temp °C	Storage Period																	
		24 hours						2 weeks						8 weeks					
		% germ	A.n.	F.e.	F.m.i.	P.f.	Others	% germ	A.n.	F.e.	F.m.i.	P.f.	Others	% germ	A.n.	F.e.	F.m.i.	P.f.	Others
A.n.	5	48	81	16	26	8	2	32	78	10	15	2	5	17	76	14	23	4	7
	20	42	84	12	21	2	6	30	76	12	14	3	8	0	78	12	18	2	6
	30	44	83	12	15	4	4	25	80	11	18	4	8	0	74	10	22	0	5
F.e.	5	40	62	72	20	5	4	36	58	72	15	4	5	20	48	68	15	6	5
	20	42	68	68	18	3	9	30	68	69	13	1	9	0	54	64	11	0	4
	30	45	58	62	12	0	6	32	72	64	12	0	8	0	56	58	12	0	4
F.m.i.	5	42	58	12	80	4	5	40	56	10	74	2	4	18	52	11	70	0	3
	20	44	48	8	78	0	4	41	46	9	72	0	6	0	66	8	68	2	2
	30	46	42	8	68	0	2	39	52	12	71	0	6	0	52	9	71	0	8
P.f.	5	44	72	8	18	32	4	41	76	10	14	8	4	35	70	10	13	8	4
	20	42	68	10	17	20	2	39	72	11	10	8	8	0	75	12	10	0	2
	30	40	67	8	13	26	6	38	82	9	9	7	10	0	80	13	2	0	12
Control	5	46	75	14	22	24	4	44	72	12	21	19	4	40	68	9	12	4	6
	20	44	81	11	19	14	10	40	74	13	18	9	8	0	69	11	11	0	2
	30	40	82	10	18	12	8	39	81	13	18	8	8	0	71	11	9	0	2

* A.n. = Aspergillus niger., F.e. = Fusarium equiseti, F.m.i. = Fusarium moniliforme var. intermedium; P.f. = Phialophora fastigiata

emerged from seeds that were inoculated with Aspergillus niger, Fusarium equiseti and Fusarium moniliforme var. intermedium produced stunted radicles. The radicles turned brown or necrotic. The seedlings which emerged from the seeds inoculated with Phialophora fastigiata and the controls appeared normal.

4.7 Location of fungi on Pinus caribaea seeds through component plating of seed parts

The testae were the main sources of fungal growth and bore 44% infection. The endosperm bore 14% and the embryo 5% (Table 10). There is a three fold increase in concentration of fungi from embryo to endosperm and to the testa. Aspergillus niger, Fusarium equiseti and Phialophora fastigiata were isolated from the endosperm and the embryo.

4.8 Location of fungi on Pinus caribaea seeds by means of scanning electron microscopy (SEM)

High quality screen and photographic pictures of the seedcoat were obtained. Some hyphae however, when placed under vacuum collapsed into a ribbon-like form. Seedcoat of Pinus caribaea was seen to be rough (Plate 10). It

Table 10. Total number of fungi identified from component parts of Pinus caribaea var. hondurensis seeds after surface sterilization in 1% NaOCl for 5 minutes (100 seeds tested).

Fungal species	Incidence of occurrence on		
	Testa	Endosperm	Embryo
Aspergillus niger	3	3	1
Chaetomium globosum	1	-	-
Fusarium moniliforme var. intermedium	2	-	-
Fusarium equiseti	12	7	2
Fusarium spp.	15	2	-
Penicillium sp.	2	-	-
Pestalotia sp.	1	-	-
Phialophora fastigiata	5	2	2
Phomopsis occulta	1	-	-
Rhizopus sp.	1	-	-
Trichoderma sp.	1	-	-
Total	44	14	5

consisted of ridges and groves on which were attached debris (possibly including spores and fungal fragments).

Examination of seeds which had been incubated for seven days before SEM showed that hyphae structures covered only the seed surface. Hyphae were found to traverse cracks on the seedcoat and do not appear to penetrate through cracks into the endosperm (Plate 11).

Examination of germinating seeds showed the emerging radicle to be clean of spores or mycelia. Even though mycelia structures were observed close to the radicle (i.e. on the seed coat adjacent to the base of the radicle) no hyphal structure was observed on the radicle (Plate 12). Plates 13 and 14 show spores and mycelia of Phialophora fastigiata and Aspergillus sp. respectively on seeds which were incubated for seven days. By comparing the spores of these fungi with the structures on the seed coat, spores of similar looking fungi were identified. Many spores which looked like those of Phialophora fastigiata and Aspergillus niger were observed on the seed coat. It was not possible to categorize those spores with certainty since the spores of many genera of fungi have similar morphology.

Plate 10: Scanning electron micrograph of the testa of Pinus caribaea var. hondurensis showing ridges and grooves for the lodgement of fungal spores.

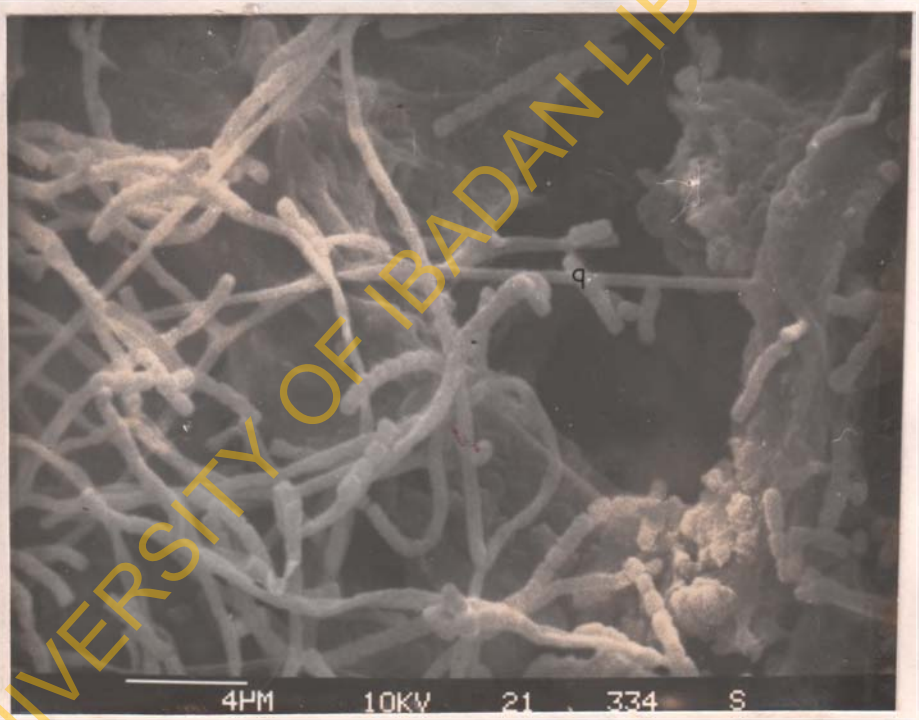

Plate 11: Scanning electron micrograph of the testa of Pinus caribaea var. hondurensis seed which had been incubated on blotter for 7 days. Note fungal hypha (q) penetrating the crack on the testa.

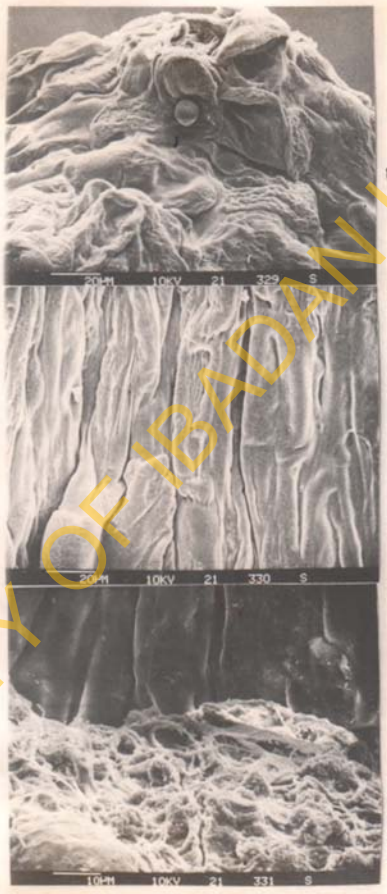

Plate 12: Scanning electron micrograph of seedling which emerged from seeds of Pinus caribaea var. hondurensis inoculated with Aspergilius niger. Mycelia grow profusely at the base of the radicle (b) and are absent on other parts of the radicle (m and t).

Plate 13: Scanning electron micrograph of spores(s) and hyphae (h) of Phialophora fastigiata on Pinus caribaea var. hondurensis testa.

Plate 14: Scanning electron micrograph of spores and hyphae of Aspergillus niger on the testa of Pinus caribaea var. hondurensis.

4.9 *In vitro studies of the fungicidal action of three plant protection compounds against some seed-borne fungi of Pinus caribaea var. hondurensis.*

In the study of the effect of three plant protection compounds, Benlate, Captan and Thiram, on the radial growth of three seed-borne fungi of *Pinus caribaea* var. *hondurensis* (*Aspergillus niger*, *Fusarium moniliforme* var.*intermedium* (*F.m.i.*), *Phialophora fastigiata*), Benlate gave complete inhibition of growth of *Phialophora fastigiata*, *F.m.i.* and *Aspergillus niger* at 1, 20 and 40 ppm respectively. Captan gave complete inhibition of the growth of *Aspergillus niger* at 40 ppm and partial inhibition of the growth of *F.m.i.* and *Phialophora fastigiata* at all tested concentrations (1 to 400 ppm). Thiram gave a complete inhibition of the growth of *Aspergillus niger* and *Phialophora fastigiata* at 20 and 400 ppm respectively and partial inhibition of the growth of *F.m.i.* at all tested concentrations (Table 11).

The analysis of variance of the mean daily radial growth of the three fungi in all the nine concentrations of the three chemicals and the control was carried out

Table 11. Effect of some fungicides on the mean radial growth (cm) of some seed-borne fungi of Pinus caribaea var. hondurensis

Seed-borne Fungi	Fungicide	(Concentrations of fungicide (ppm)									
		0	1	2	4	10	20	40	100	200	400
F.m.i.	Benlate	4.2	3.4	1.8	0.2	0.1	0.0	0.0	0.0	0.0	0.0
	Captan	4.2	4.2	4.0	3.8	2.4	1.9	1.3	0.9	0.7	0.4
	Thiram	4.0	3.9	3.9	3.6	1.6	1.0	0.6	0.4	0.3	0.2
P.f.	Benlate	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Captan	2.0	1.8	1.6	1.6	1.5	1.4	1.3	0.8	0.7	0.5
	Thiram	2.2	2.3	1.8	0.9	0.8	0.6	0.6	0.4	0.3	0.0
A.n.	Benlate	2.8	2.7	2.6	2.5	1.8	1.1	0.0	0.0	0.0	0.0
	Captan	2.7	2.7	2.6	2.1	1.4	1.1	0.0	0.0	0.0	0.0
	Thiram	2.6	2.6	2.5	2.2	1.7	0.0	0.0	0.0	0.0	0.0

F.m.i. = Fusarium moniliforme var. intermedium
P.f. = Phialophora fastigiata
A.n. = Aspergillus niger., F.e. = Fusarium equiseti.

after square root transformation of the raw figures (Appendices 3, 4 and 5). The results (Table 12) showed that all the chemicals and all the nine concentrations had significant effects on the growth of all the fungi. It also showed that interactions such as chemical/fungi, chemical/concentration, fungi/concentration and chemical/fungi/concentration were significant at 0.01%. This analysis showed that Benlate was the most superior in reducing the growth of all the fungi (mean of 0.96 cm/day), followed by Captan (mean 1.23 cm/day) and then Thiram (mean 1.39 cm/day). The fungus Phialophora fastigiata was the most affected (mean of 1.05 cm/day), followed by Aspergillus niger (mean 1.17 cm/day) and lastly Fusarium moniliforme var. intermedium (mean 1.35 cm/day).

Duncan Multiple Range test was carried out to bring out differences between the various means. This showed that the effect of Benlate on Phialophora fastigiata was the most superior compared with the other treatments which were lumped together. Its mean was 0.7071 cm/day compared

Table 12. Analysis of variance of mean radial growth rate of 3 fungi (*Aspergillus niger*, *Fusarium moniliforme* and *Phialophora fastigiata*) in PDA impregnated with 9 doses of fungicides (Benlate, Captan and Thiram).

(Statistical analysis of the results presented in Appendix 5 to 7)

Source of variation	D.F.	Sums of square	Mean Sq.	F
Main effects	12	52.179	4.348**	1087.00
Fungicides	2	10.210	5.105**	1276.25
Fungi	2	4.778	2.389**	597.25
Dose	8	37.191	4.649**	1162.25
2-way-inter.	36	16.741	0.465**	116.25
Fungi	4	6.316	1.579**	394.75
Dose	16	2.801	0.175**	43.75
Dose	16	7.624	0.476**	119.00
3-way-inter.	32	3.756	0.117**	29.25
Fungicide/Fungi/D.	32	3.756	0.117**	29.25
Explained	80	72.676	0.908**	227.00
Residual	243	0.877	0.004	
Total	323	73.553	0.224	

**Significant at 0.01 level of probability.

with the means of others which ranged from 0.9562 - 2.1 (Table 13). Captan and Thiram affected Phialophora fastigiata equally and significantly suppressed the growth of this fungus.

Significant suppression of F.m.i was got with Benlate compared with Captan, Thriam and Control. Also Captan and Thiram gave significant reduction in growth of the fungus.

Benlate, Captan and Thiram have equal effects on the growth of Aspergillus niger causing a significant reduction in growth.

Generally, all the three fungicides led to a reduction in growth of all the fungi. On the growth of Aspergillus niger, all the three fungicides had almost the same reduction effect at increasing concentration (Fig. 1). Benlate led to the greatest reduction in growth of Fusarium moniliforme var. intermedium followed by Thiram and Captan (Fig. 2). Benlate stopped the growth of Phialophora fastigiata at very low concentration of 1 p.p.m. while the fungus was still able to grow in higher concentrations of Thiram and Captan (Fig. 3).

Table 13. Effect of Benlate, Captan and Thiram on the mean growth (± S.E.) of *Aspergillus niger*, *Phialophora fastigiata* and *Fusarium moniliforme* var. *intermedium* (F.m.i) isolated from the seeds of *Pinus caribaea* var. *hondurensis*

Group	Treament	Mean
1.	Benlate/A. *niger*	1.2107 ± 0803a
2.	Benlate/F.m.i.	0.9562 ± 0.0760b
3.	Benlate/P. *fastigiata*	0.7071 ± 0.0004f
4.	Captan/A. *niger*	1.1829 ± 0.0773a
5.	Captan/F.m.i.	1.6742 ± 0.0761e
6.	Captan/P. *fastigiata*	1.3061 ± 0.0332ac
7.	Thiram/A. *niger*	1.1329 ± 0.0816ab
8.	Thiram/F.m.i.	1.4138 ± 0.0856dc
9.	Thiram/P. *fastigiata*	1.1427 ± 0.0687ab
10.	Control/A. *niger*	1.7913 ± 0.0268e
11.	Control/F.m.i.	2.1244 ± 0.1975g
12.	Control/P. *fastigiata*	1.5938 ± 0116de

Note: Means followed by the same letter in the column are not significantly different at 0.01 level of probability (DMRT).

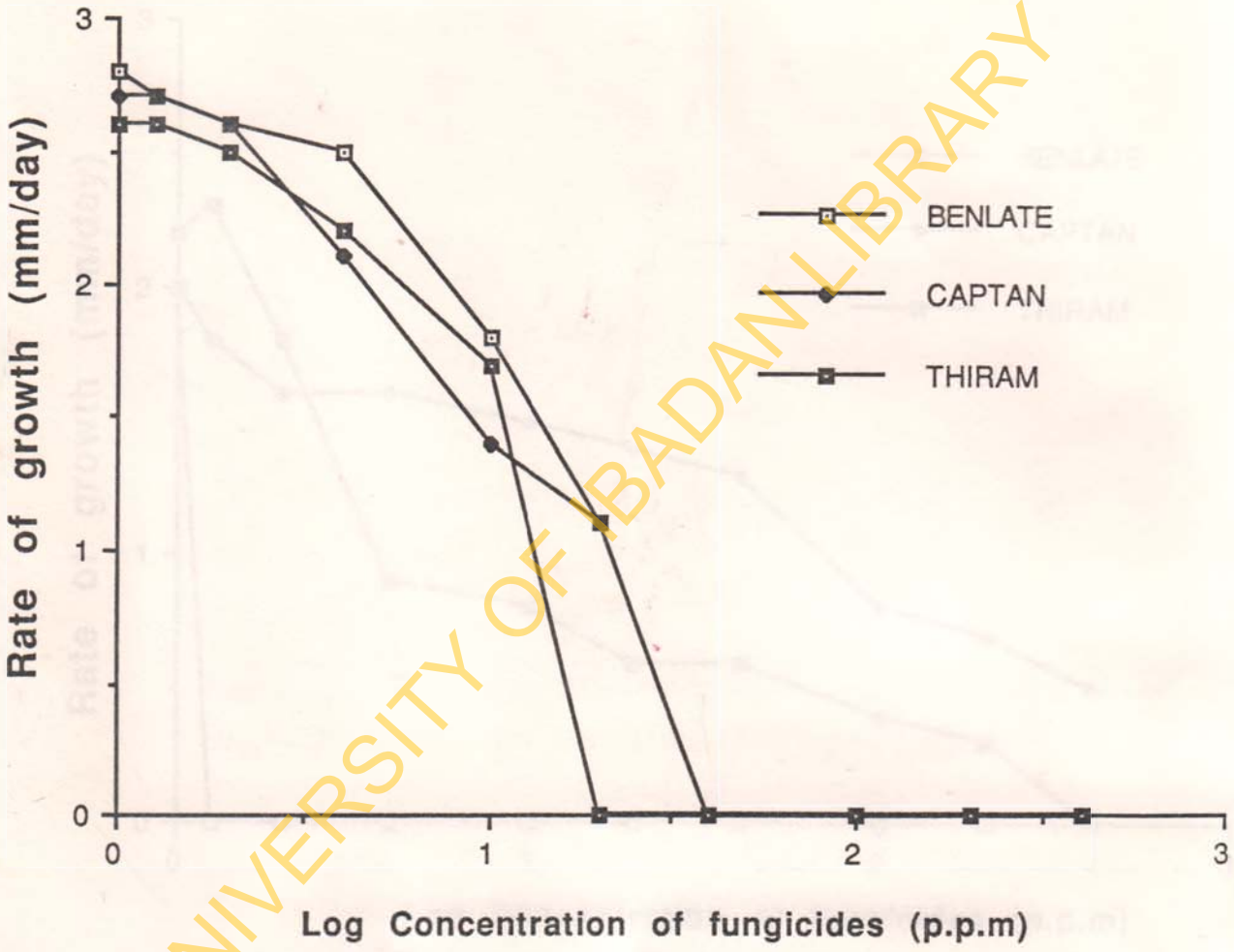

fig 1 : Rate of growth Aspergillus niger **with various concentrations of Benlate, Captan and Thiram.**

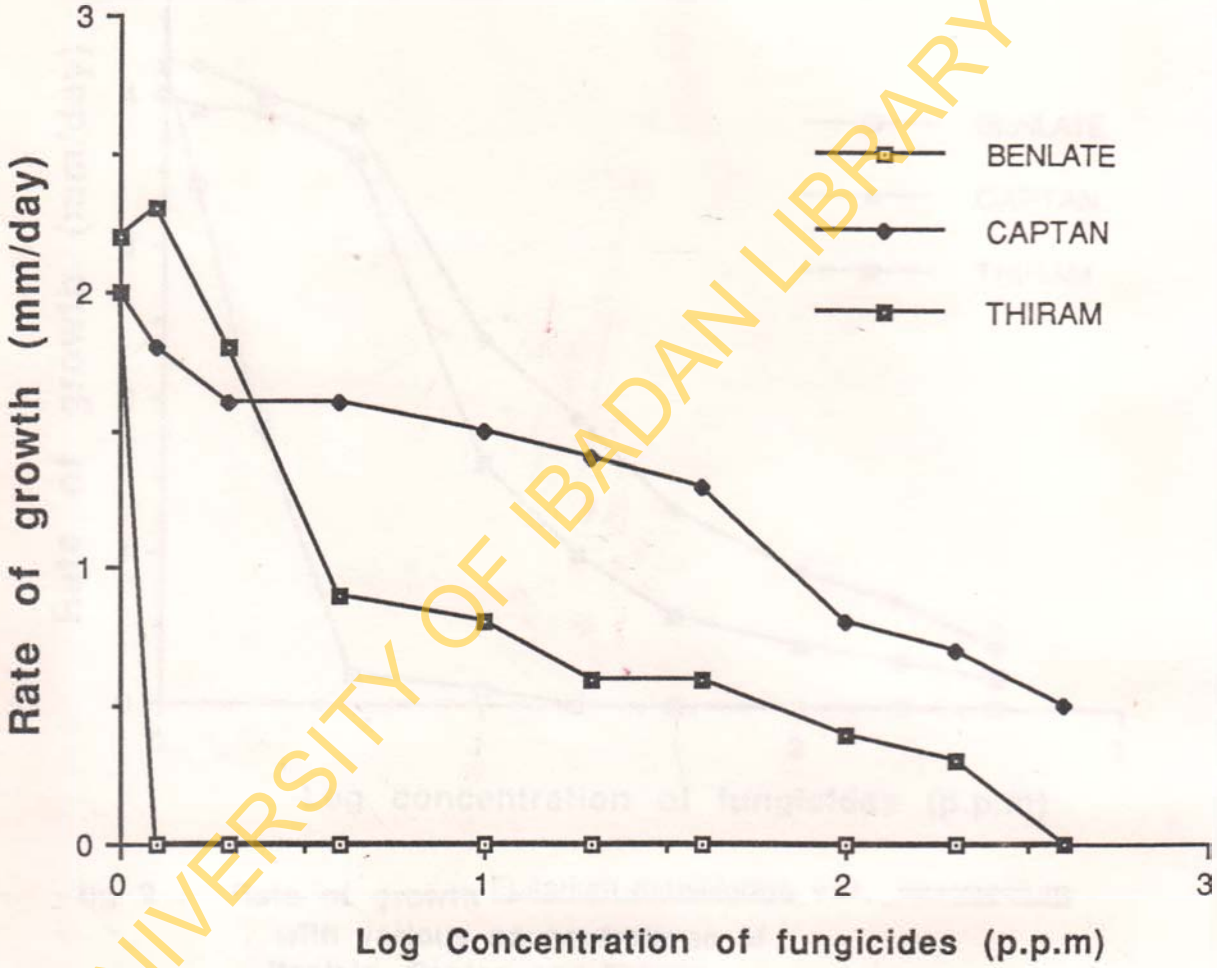

fig 3 : Rate of growth phialophora fastigiata with various concetrations of Benlate, Captan and Thiram.

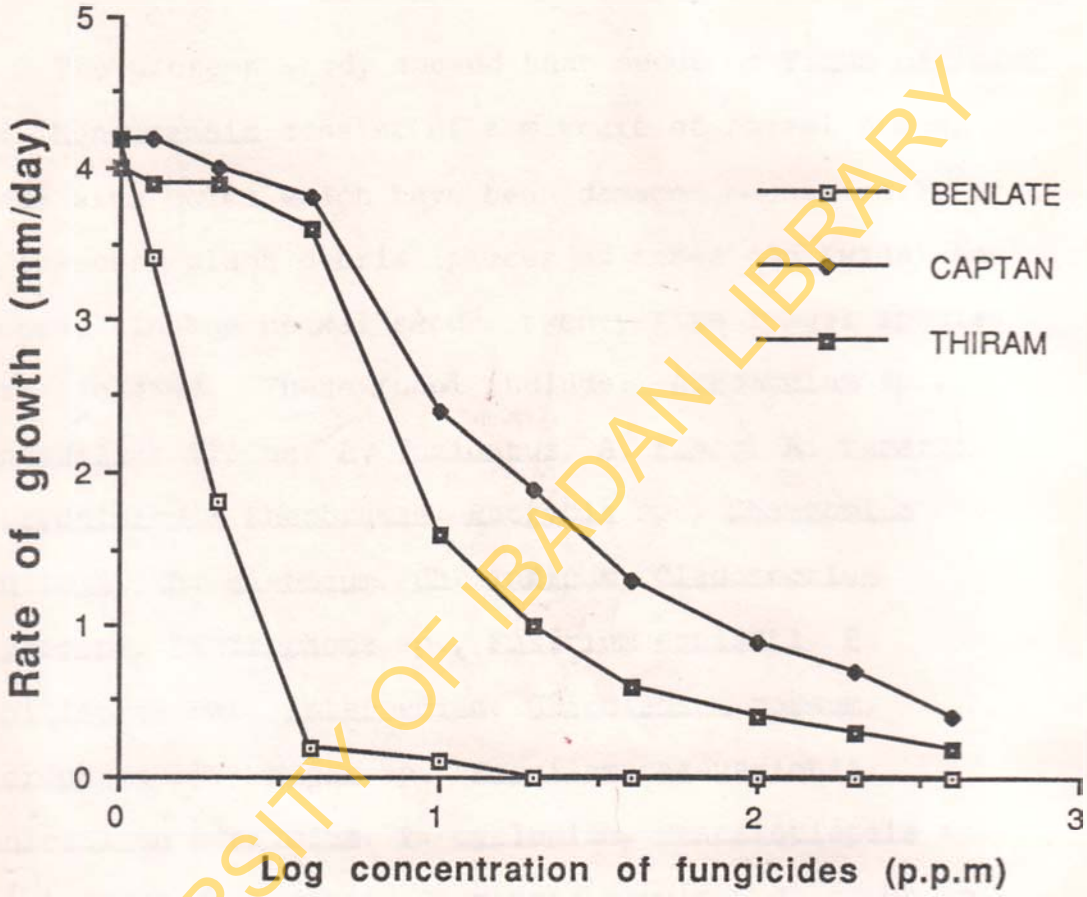

fig 2 : Rate of growth Fusarium moniliforme var. intermedium **with various concentrations of Benlate, Captan and Thiram.**

CHAPTER FIVE

DISCUSSION

The present study showed that seeds of Pinus caribaea var. hondurensis consist of a mixture of normal seeds, seeds with holes which have been damaged mechanically or by insects, plant debris (pieces of cones and twigs) and stones. In the normal seeds, twenty five fungal species were isolated. These fungi include: Acromonium sp., Aspergillus flavus, A. fumigatus, A. niger, A. tamarii, Botryodiplodia theobromae, Botrytis sp., Chaetomium funicola, Ch. globosum, Ch. indicum, Cladosporium oxysporum, Dendrophoma sp., Fusarium equiseti, F. moniliforme var. intermedium, Gliocladium roseum, Macrophoma sp., Mucor sp., Paecilomyces variotii, Penicillium citrinium, P. cyclopium, Pestalotiopsis sp., Phialophora fastigiata, Phomopsis occulta, Rhizopus sp. and Trichoderma sp. A large proportion of the identified fungi are facultative pathogens according to Booth (1971) and Hawksworth et al. (1983). Notable among these pathogens are: Aspergillus. fumigatus, A. niger, A. tamarii, Botryodiplodia theobromae, Cladosporium oxysporum, Fusarium equiseti, F. moniliforme var. intermedium, Penicillium citrinium, Phialophora fastigiata

and Phomopsis occulta. According to Fatuga (1973), A. niger, A. flavus, Mucorales, F. moniliforme, Fusarium sp., F. semitectum and Penicillium sp. were isolated from seeds of P. caribaea. More fungi species were isolated in the present study compared with that of Fatuga (1973) perhaps because more samples were examined. However, Rees (1983) detected 155 fungal species from various tropical pine seeds such as P. caribaea, P. oocarpa, P. pseudostrobus out of which 27 were plant pathogens. Although no study was conducted to trace the sources of the fungi, Whittle (1977) found that Sclerophoma pythiophila and Penicillium spp., which constituted the entire mycoflora of seeds of P. sylvestris were from the extraction unit and the air. It might therefore be possible that a large proportion of the fungi isolated in this study originated from similar sources. Mathur (1984) made similar observation by reporting the presence of Botryodiplodia sp., B. theobromae, Botrytis cinerea, Chaetomium sp., Drechslera bicolor, F. moniliforme, F. oxysporum, F. semetectum, F. solani, Macrophoma phaseolina and Pestalotia sp. on seeds of P. caribaea.

The most frequently occurring fungi in this study were A. niger, Chaetomium globosum, F. equiseti, F.

moniliforme var. intermedium, Gliocladium roseum and Phialophora fastigiata. A. niger with the highest frequency of occurrence has been confirmed in the present study to be a pathogen of seeds of P. caribaea. It resulted in 60% decrease in germination percentage and formation of abnormal seedlings. Mittal (1983) noted that A. niger led to the production of brownish discoloration at the joint of needles with the stem and eventually led to rotting of the stems in seedlings of P. roxburghii Sarg. Munjal and Sharma (1976) working on the mycoflora of some important conifers in India showed that A. flavus, A. niger, Coniothecium atrum, Curvularia spp., Helminthosporium sp., Oedocephalum glomerulosum, Penicillium sp., Phoma hibernica, Sordaria fimicola and Stemphylum botryosum caused considerable pre-emergence losses of both P. roxburghii and P. wallichiana while Cephalosporium sp., Fusarium spp., Mucor spp. and Trichoderma viride caused heavy post-emergence losses on the two conifers.

The observation that insect and mechanically damaged seeds harboured seed borne pathogens such as A. flavus, A. niger, F. moniliforme var. intermedium and Phomopsis occulta and that the debris carried fungi like A. niger,

Chaetomium globosum, F. equiseti, F. moniliforme var. intermedium, Macrophoma spp., Pestalotiopsis spp. and Phialophora fastigiata showed that damaged seeds and debris provided favourable conditions for the proliferation of the pathogens. This conforms with earlier reports by Ralph (1977) that the micro-organisms carried on the seed debris and the damaged seeds subsequently contaminated the seeds. McGee (1983) noted that insect damages on seeds serve as entry points for micro-organisms and lead to seed deterioration. Also insects have been found to transfer fungus carried on or within them into seeds through the ovipositor and cracks in the testa (Mills, 1983). Rowan and DeBarr (1974) isolated Fusarium solani from seedbug-damaged seeds and such seeds showed a lower germination compared with the control. Since the seed debris and insect/mechanically damaged seeds are unavoidably mixed with normal seeds, then the role they play in the dissemination of important diseases of pines especially across international boundaries should not be overlooked.

All the pine seeds in this study carried pathogenic organisms which are known to reduce seed germination and seedling growth. Their presence could lead to pre and

post emergence mortality diseases in the nursery and the plantation and contamination of the soil. Other crops could eventually be infected when planted in an area where the pathogen had been introduced and this might lead to the introduction of new diseases into the country.

In the present study, the blotter and agar techniques appeared to be the best methods for the detection of seed borne fungi on pine seeds. The direct inspection of dry seeds was not accurate in that in most cases seeds which were discoloured and suspected to harbour fungal structures turned out to be "clean" or healthy after incubation on agar. This observation is contrary to that of Brown (1984), that pine seeds classed into healthy, discoloured and/or shrunken, and replaced by thick fungal tissue yielded on agar 11.7%, 45.8% and 91.3% B. theobromae respectively, suggesting the involvement of this fungus in seed deterioration. Anderson (1986b) working on a rapid method for the isolation of F. moniliforme var. subglutinans from pine seeds modified the blotter and agar methods to the blotter-crushed seed method. This he did by placing the seed on blue filter paper in a plastic tray, crushing the seed, and spraying the seed and the blotter paper with a liquid medium

semiselective for Fusarium spp. The tray was covered and incubated at room temperature (20°C) for 14 days or until the colonies were 2 cm in diameter. This method permitted rapid screening of 25 seeds at a time compared with 5 seeds by the agar method and it saved the cost of the agar.

The reduction in the isolation of some fast growing fungi like A. niger and Rhizopus sp. due to surface sterilization in 1% sodium hypochlorite for 5 minutes also confirms earlier studies by Sutherland and Woods, 1978. Rees, 1983 noted that the treatment of pine seeds in 6% sodium hypochlorite for 5 minutes was effective in reducing the isolation of A. niger but was not effective for B. theobromae, Penicillium sp. and Schizophyllum commune. This treatment enabled the observation of some slower growing fungi such as B. theobromae and Cladosporium oxysporum. The observation that A. niger and Rhizopus sp. were isolated more from untreated seeds than from surface sterilized seeds showed that these fungi occurred mainly as surface contaminants.

The differences in the incidence of micro-organisms between the seed lots could be attributed to a combination of factors. Among these are the geographical location of

each seed lot, the weather at the time of collection and different extraction techniques. Sutherland and Woods (1978) found that more seeds were infected with Calosypha fulgens after manual processing when compared with machine extraction.

The seedling symptom tests showed that disease symptoms were developed at the seedling stage. Disease symptoms of damping-off were observed in both the control (unsterilized seeds planted in sterilized soil) and the treated (surface-sterilized seeds planted in sterilized soil). But more seedlings (10%) were diseased in the control. This result indicated that the disease causing organisms were in or on the seeds and that surface sterilization of the seeds could not eliminate them all.

The results of inoculation of seeds with A. niger, F. equiseti and F. moniliforme var. intermedium showed that these organisms can infect seeds of P. caribaea var. hondurensis. Such infection led to reduction in germination of the seeds and the emerging seedlings showed abnormal growth. Previous reports have shown similar observation with different pine species (Urosevic, 1964; Mittal, 1983 and Rees, 1983). The profuse growth of the pathogens in inoculated seeds and the re-isolation of the

pathogens from dead seeds and abnormal seedlings confirmed Koch's postulate. A. niger caused stunted radicle growth similar to an earlier report by Fisher (1941). This fungus is known to produce oxalic acid which is toxic to plants (Gibson, 1953). Phialophora fastigiata had no effect on seed germination or seedling development.

On trials of various inoculation techniques of seedlings, all the four tested methods (seedling plate technique; root-inoculation with fungi discs; stem inoculation by wounding and shoot inoculation by spore spray) were successful in proving that the tested fungi were pathogenic. The seedling plate technique was a rapid way of detecting pathogenicity of fungi isolated from seeds. F. equiseti and F. moniliforme var. intermedium caused pre-emergence death and seedling death. Whenever the roots were inoculated by any of these fungi, the roots became necrotic, the stem and needles wilted and eventually the seedling died. The seedlings that were inoculated by wounding and injecting inoculum into the wound, showed necrotic growth at the inoculated area and the seedling eventually wilted and died. The seedlings which were inoculated by spore spray on the shoots first developed needle wilt and eventually died. These two

pathogenic fungi grew profusely in areas where they were put on the seedlings, they were re-isolated from dead seedlings thus proving Koch's postulate. A. niger and Phialophora fastigiata had no effect on inoculated seedlings. The results on the inoculation trials agreed with that of Chou (1976b) where 50-80% infection was obtained in seedlings which were inoculated by injecting fungi spore suspension into wounds made artificially on pine seedlings. He noted that the fungi led to reduced seedling growth and that the mode of action of the fungi probably involved a toxin that inhibited seedling growth. His observation also indicated that wounds and insects damage can predispose pines to infection by creating easy access for plant pathogenic micro-organisms in the environment to get in and cause death.

F. moniliforme var. intermedium the most pathogenic fungus examined is known to cause tree diseases all over the world (Labrada, 1973; Dwinell, 1978). All the pine seeds and seedlings were susceptible to its attack. This was the expected result for seeds with light coloured seed coats such as the pines used in this study. Crzywacz and Rosochacka (1977, 1980) noted that light coloured seeds contain less amount of erucic acid (a toxin to some fungi)

and so they are less resistant to pre-emergence damping-off fungi.

On the effect of storage temperature on germination of seeds and invasion of fungi, it was shown that seeds would maintain their initial germination for sometime if stored at a low temperature of 5°C whereas the germination would drop if the seeds are stored at higher temperatures of 20° and 30°C. The viability of the inoculated and non-inoculated seeds stored at 20° and 30°C reduced drastically to zero by the 8th week of storage. The uninoculated seeds which were stored at 5°C maintained the initial percent germination of 48% whereas this dropped to 17-20% in seeds which were inoculated with A. niger, F. equiseti and F. moniliforme var. intermedium. This observation agreed with that of McGee (1983), that ageing and invasion by micro-organisms and insects are the main causes of seed deterioration. Neergaard (1977) and Anderson and Baker (1983) noted that seeds should be stored in sealed containers at 0-5°C because such properly stored seeds can remain viable and vigorous for many years. Since the temperature of the tropical region (including Nigeria) is between 20-30°C during the day, this high temperature may be responsible for the poor

germination of our pine seeds which are stored in ordinary room temperature when electricity is not available in the store. The occurrence of A. niger remained high at 5°, 20° and 30°C throughout the duration of this study. According to Sutherland (1979), most tree seeds become contaminated during processing when the seeds are removed from the cones and the micro-organisms continue to increase in number while the seed is in storage.

The majority of fungal isolates came from the seedcoat alone. According to Halloin (1983), many of these fungi would not be able to invade the seeds as the seedcoat is known to form an external barrier to fungal invasion. Very few isolates were present on the seedcoat, the endosperm and the embryo. No isolate was obtained from the endosperm and or embryo alone contrary to the findings of Rees (1983). This contradicts Anderson et al.(1984) in the United States of America who isolated F. moniliforme, Diplodia sp., and Fusarium sp. from the inside of P. elliottii. Fungal isolates from pine seeds depend on the maturity of the cones and the storage period of the seeds (Brown, 1984). These factors may therefore account for the differences in fungal isolates in the

present study and that of Rees (1983), Rees and Phillips (1986).

The success of seed health testing methods used in these investigations depended on the ability of the seed-borne micro-organisms to grow on an artificial medium. Obligate pathogens and slow growing facultative parasites would not have been detected by the basic plating techniques. So experiments using the scanning electron microscope (SEM) were devised. The SEM provided direct method for observing spores and other propagules on seeds. This technique relied on the recognition of characteritic spore shapes but could not differentiate between viable and dead propagules. Through the SEM it was observed that the seedcoat of Pinus caribaea var. hondurensis was rough and could carry fungal fragments and spores. By comparing some of the structures with the prepared known fungus, spores that looked like those of Phialophora sp. and Macrophoma sp. were observed. However, this method has a limited value in that only one seed could be scanned at a time and specimen prepartion was time consuming.

In an attempt to study spore germination and penetration of the germ tubes into pine seedlings using the scanning electron microscope, hyphae were observed to

spread on the surface of the testa and were not seen to penetrate it.Perhaps the fungi were just growing as saprophytes on the seedcoat or perhaps the developing seedling exuded inhibiting chemicals to keep the pathogen off. Bloomberg (1966) supported this observation when he reported that spores and mycelia do not appear to penetrate into the seed coat of pines. Patton and Spear (1978) on Schirrhia infection of pines with scanning electron microscope found that germ tubes usually grew appressed to the needle surface and followed the contours of the epidermis. It will be premature to make conclusive deduction on fungal penetration using the scanning electron microscope in the present investigation. More detailed studies would therefore be necessary. It might be required that inoculated seeds and seedlings at very early stage of infection would have to be sectioned and examined with the light and scanning electron microscopes.

The result of the effect of Benlate, Captan and Thiram on the growth of A. niger, F. moniliforme var. intermedium and Phialophora fastigiata showed that some control of the fungi was possible through the use of these chemicals. Benlate at 1 ppm was the best in inhibiting the growth of Phialophora fastigiata, followed by Thiram

at 400 ppm while Captan did not inhibit the growth of this fungus. Thiram was the most effective in inhibiting the growth of A. niger at 20 ppm, while Benlate and Captan were equally effective at 40 ppm. Benlate was the only effective chemical on F. moniliforme var. intermedium at 20 ppm. Hence Benlate is recommended in the control of F. moniliforme var. intermedium and Phialophora fastigiata. In the control of the three fungi a combination of Benlate and Thiram is recommended. Any one of the three chemicals, Benlate, Captan or Thiram could be used in the control of A. niger. Different chemicals have been recommended by various authors for the control of fungal diseases in seeds and seedlings of pines. Mittal and Sharma (1982) in a comparative study of the effect of nine fungicides on the control of some seed-borne fungi of P. wallichiana reported that Thiram and Dithane M-45 were the best fungicides, as only a single fungal species developed on the tested seeds, while Agrosan GN, Brassicol, Ceresan, Captan, RH-2161 and Bavistin were also good since they reduced the number of developing fungi, and Penoctine-35% was the least effective. The present observation that a combination of two or more chemcials would be effective in the control of some seed-borne fungi of P. caribaea var.

hondurensis confirms the work of Wall, (1976) cited in Odeyinde and Ofong (1984). The author noted that since Benlate was effective for the control of Fusarium spp. and ethazole for Pythium spp., a combination of the two chemicals was recommended in damping-off control. Vaartaja (1964) also observed that a single chemical can not be recommended for the prevention of damping-off because of the diversity of the fungi involved. On the contrary, Hocking and Jaffer (1969) found that Rhizoctol applied in a sticker (4 percent hydroxypropyl methyl cellulose) was the only fungicide which effectively controlled damping-off and gave increased stands of seedlings. Munjal and Sharma (1976) showed that Agrosan (Phenyl mercury acetate plus ethyl mercury chloride) and Cerosan (Phenyl mercury acetate) at 0.25% concentrations inhibited completely the growth of A. niger and some other seed-borne fungi. Fatuga (1973) reported that Agrosan 5W (8.1% Totylmercury acetate) had no significant effect on the seed germination of P. caribaea but its effect on the growth of seed-borne organisms was not reported by the author.

The use of chemicals in the control of seed-borne fungi of pines must be done with caution. The phytotoxic

effect of Captan to seed and seedlings of pines has been reported by many workers. Kozlowski (1986) noted that Captan, at concentration up to 2,500 ppm did not affect seed germination of Pinus resinosa Ait. However, concentrations of 500 ppm or higher injured roots, stems, and cotyledons within 13 days. His observation is consistent with data of other studies showing that Captan was phytotoxic and could injure leaves, fruits and also inhibit seedling growth (Daines et al., 1957; Denne and Atkinson, 1973; Gram and Vaartaja, 1957 and Vaartaja, 1964).

From the above it can be concluded that repeated fungicide applications to young pine seedlings increase the risk of chemical injury, and could affect the microbial equilibrium in the rhizosphere (Vaartaja, 1964). Mycorrhizal formation could also be retarded (Bakshi and Dobriyal, 1970). The chemicals recommended for the prevention of damping-off may not be effective against all pathogens (Vaartaja, 1964). Hence the fungicide for each pathogen has to be worked out and a mixture of fungicides tried in the damping-off complex (Wall, 1976). It is also necessary to establish the effective dose of chemical that would eliminate the fungus without injuring the seedling.

CHAPTER SIX

SUMMARY AND CONCLUSION

Seeds of Pinus caribaea var. hondurensis were examined by dry inspection, blotter and agar-plate methods for the presence of seed-borne fungi. Twenty-five species of fungi from eighteen genera were isolated and their identification confirmed by the Commonwealth Mycological Institute. The seed-borne fungi were mainly carried on the testa. This was confirmed through component plating of seed parts and scanning electron microscopy (SEM). Seed debris (twigs and cone pieces) stones and dead insects also carried some seed-borne fungi.

Examination of the seeds by SEM showed that the testae were rough and carried structures which looked like fungal spores and mycelia. SEM of germinating seedlings revealed that fungal structures were not seen on the radicle, but only on the testa.

Seedling symptom test showed damping-off and seedling blight to be the only diseases of seeds and seedling of Pinus caribaea var. hondurensis. The associated fungi were Botryodiplodia theobromae, Fusarium moniliforme var. intermedium, Rhizoctonia spp. and Rhizopus spp.

Pathogenicity studies indicated that Aspergillus niger, Fusarium equiseti, F. moniliforme var. intermedium led to drastic reduction in seed germination whereas Phialophora fastigiata seemed to have little or no effect on seed germination. Seeds which were inoculated with A. niger, F. equiseti, F. moniliforme var. intermedium and the controls when stored at 20° and 30°C lost their viability within eight weeks (a drop of 40 to 0%). The inoculated seeds kept at 5°C gave a drop of 40 to 17%. The uninoculated seeds maintained their initial percentage germination of 40% when kept at 5°C. Hence cold temperature is essential for the maintenance of viability.

Seedlings which emerged from seeds inoculated with A. niger, F. equiseti, F. moniliforme var. intermedium produced stunted radicles. The health of 3-weeks and 3-months old seedlings inoculated with A. niger and Phialophora fastigiata appeared not to be disturbed. Whereas F. equiseti, F. moniliforme var. intermedium led to wilting of seedlings and seedling mortality.

Histological studies of healthy and infected stem sections stained with aniline blue in lactophenol showed that fungi were present in all the cells; hyphae

penetrated and blocked the tracheids while parenchyma cells were disintegrated.

In vitro studies of the effect of Benlate, Captan and Thiram on the control of some frequently isolated fungi (A. niger, F. moniliforme var. intermedium and Phialophora fastigiata) indicated that Benlate was superior in suppressing the growth of Phialophora fastigiata and F. moniliforme var. intermedium while both Captan and Thiram had equal suppresing effect on the two fungi. But Benlate, Captan and Thiram were equally effective in reducing the growth of A. niger.

In conclusion therefore:

1. Pinus caribaea var. hondurensis seeds should be stored in an area where temperature is maintained at 5°C or below.
2. When Pinus caribaea var. hondurensis seeds are to be planted, seeds with holes due to insect or mechanical damage, stones, plant debris should be picked and discarded.
3. Seeds to be planted should be surface sterilized in 1% sodium hypochlorite for 5 minutes in order to get rid of some storage fungi.

4. The seeds should be treated with fungicides like Thiram or Benlate in order to reduce drastically the effect of damping-off organisms.

5. Damped-off seedlings should be pulled out and discarded to reduce the spread of the disease.

6. The routine sterilization of the germination and sowing media already in practice in Forestry Research Institute and other pine growing nurseries should continue.

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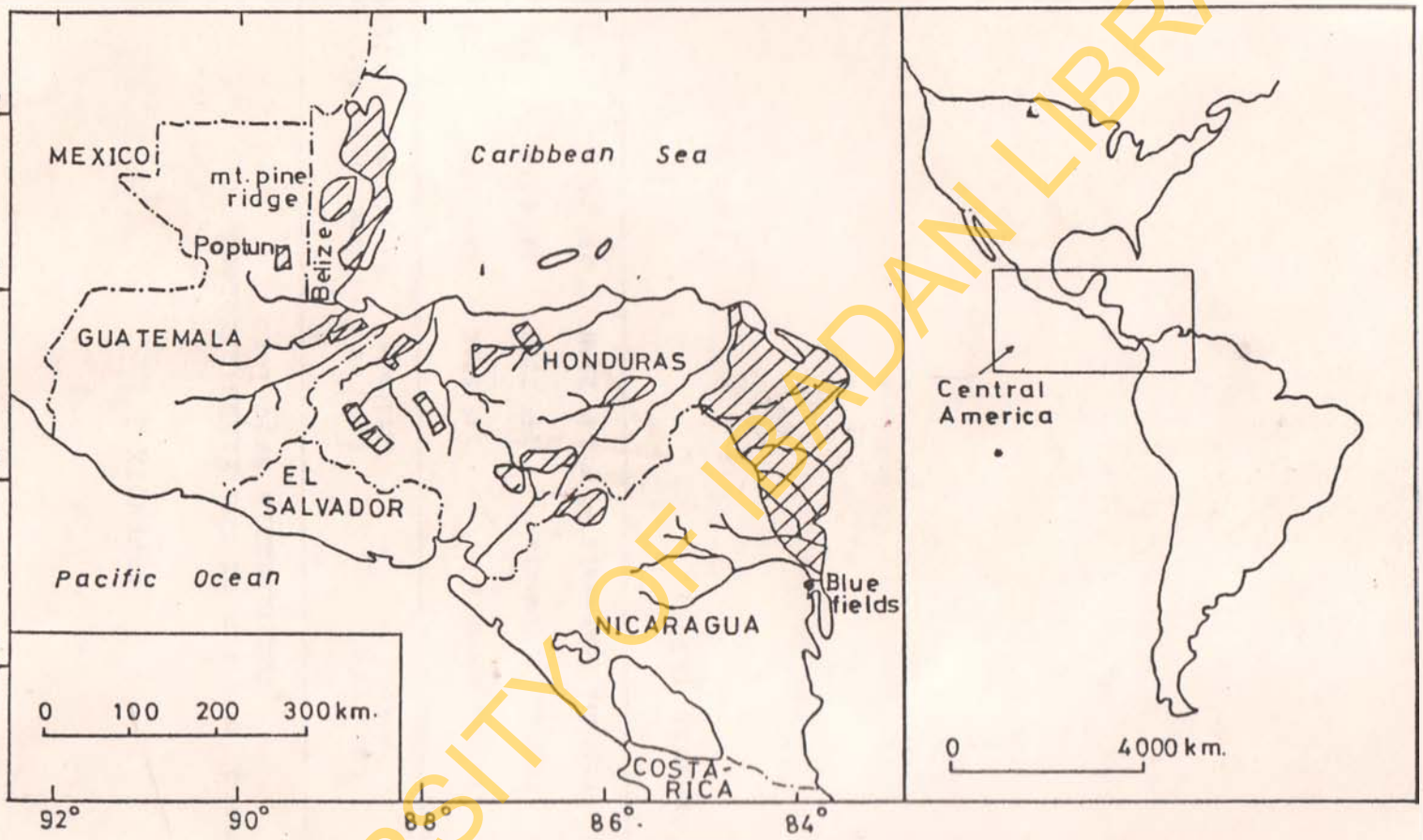

The natural distribution ▨ of Pinus caribaea var. hondurensis Barr. and Golf (adapted from Greaves, 1980).

APPENDIX 2

Stand location of the Pinus caribaea var. hondurensis seed lots

Index No	Country of origin	Lat. or latitude	Long. or longitude	Att. or Attitude
12/81	Yojoa, Hondura	14° 58'N	88° 15'W	479 m
50/78	Poptun, Guatemala	16° 21"N	89° 25'W	500 m
7139	Poptun, Guatemala	16° 21'N	89° 25'W	500 m

Source: Greaves (1978).

APPENDIX 3

The radial growth of Aspergillus niger in Benlate, Captan and Thiram

TREATMENTS			REPLICATES			
Fungi	Chemical	Conc. (ppm)	I	II	III	IV
Aspergillus niger	Benlate	0	2.9	2.9	2.5	3.0
		1	2.5	3.1	2.6	2.6
		2	2.5	2.6	2.6	2.5
		4	2.5	2.5	2.4	2.5
		10	2.5	1.8	1.6	1.6
		20	0.9	1.3	1.0	1.3
		40	0.0	0.0	0.0	0.0
		100	0.0	0.0	0.0	0.0
		200	0.0	0.0	0.0	0.0
		400	0.0	0.0	0.0	0.0
	Captan	0	2.5	2.8	2.9	2.8
		1	2.6	3.0	2.5	2.6
		2	3.0	2.5	2.6	2.3
		4	2.1	1.9	2.0	2.5
		10	1.6	1.3	1.3	1.6
		20	1.5	0.5	1.0	1.5
		40	0.0	0.0	0.0	0.0
		100	0.0	0.0	0.0	0.0
		200	0.0	0.0	0.0	0.0
		400	0.0	0.0	0.0	0.0
	Thiram	0	3.1	2.3	2.9	2.0
		1	2.5	2.9	2.9	2.3
		2	2.1	2.8	2.8	2.3
		4	2.3	2.3	2.3	2.1
		10	1.8	1.8	1.6	1.8
		20	0.0	0.0	0.0	0.0
		40	0.0	0.0	0.0	0.0
		100	0.0	0.0	0.0	0.0
		200	0.0	0.0	0.0	0.0
		400	0.0	0.0	0.0	0.0

APPENDIX 4

The radial growth of Fusarium moniliforme var.intermedium (F.m.i.) in Benlate, Captan and Thiram

TREATMENTS			REPLICATES			
Fungi	Chemical	Conc. (ppm)	I	II	III	IV
F.m.i.	Benlate	0	4.0	3.8	3.6	3.8
		1	3.5	3.4	3.5	3.4
		2	1.3	1.9	2.0	2.3
		4	0.0	0.0	0.6	0.0
		10	0.0	0.0	0.3	0.0
		20	0.0	0.0	0.0	0.0
		40	0.0	0.0	0.0	0.0
		100	0.0	0.0	0.0	0.0
		200	0.0	0.0	0.0	0.0
		400	0.0	0.0	0.0	0.0
	Captan	0	4.5	4.3	4.1	4.0
		1	4.3	4.1	4.3	4.1
		2	4.0	3.8	4.1	4.0
		4	4.0	3.8	3.5	3.8
		10	3.0	3.6	3.5	3.4
		20	3.0	3.2	3.0	2.4
		40	1.9	2.5	2.4	2.5
		100	0.9	0.9	0.9	0.9
		200	0.6	0.6	0.8	0.6
		400	0.5	0.5	0.3	0.5
	Thiram	0	4.1	4.3	3.9	3.8
		1	3.9	3.9	4.1	3.8
		2	4.0	4.0	3.9	2.9
		4	3.4	3.9	3.8	3.5
		10	1.9	1.5	1.6	1.5
		20	1.0	0.9	1.0	1.3
		40	0.6	0.5	0.9	0.3
		100	0.5	0.3	0.5	0.5
		200	0.3	0.4	0.4	0.4
		400	0.3	0.1	0.1	0.3

APPENDIX 5

The radial growth of *Phialophora fastigiata* in Benlate, Captan and Thiram

TREATMENTS			REPLICATES			
Fungi	Chemical	Conc. (ppm)	I	II	III	IV
Phialophora fastigiata	Benlate	0	2.0	2.1	2.0	1.9
		1	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0
		4	0.0	0.0	0.0	0.0
		10	0.0	0.0	0.0	0.0
		20	0.0	0.0	0.0	0.0
		40	0.0	0.0	0.0	0.0
		100	0.0	0.0	0.0	0.0
		200	0.0	0.0	0.0	0.0
		400	0.0	0.0	0.0	0.0
	Captan	0	2.0	1.9	2.0	2.0
		1	1.6	2.1	1.8	1.8
		2	1.4	1.8	1.8	1.8
		4	1.5	1.6	1.4	1.8
		10	1.5	1.4	1.5	1.5
		20	1.4	1.3	1.5	1.5
		40	1.3	1.4	1.4	1.4
		100	0.8	1.1	0.8	0.6
		200	0.5	0.5	0.6	0.5
		400	0.5	0.5	0.3	0.4
	Thiram	0	2.1	2.1	2.0	2.4
		1	2.5	2.3	2.3	2.1
		2	1.8	1.8	1.9	2.0
		4	0.8	0.9	0.9	1.3
		10	0.8	0.8	0.8	0.8
		20	0.5	0.5	0.8	0.8
		40	0.8	0.5	0.6	0.5
		100	0.6	0.5	0.4	0.3
		200	0.3	0.3	0.5	0.3
		400	0.0	0.0	0.0	0.0