SOME ASPECTS OF THE ECOLOGY AND PATHOLOGY OF

PHAEOSEPTORIA EUCALYPTI HANS. EMEND.

WALKER ON SOME SPECIES OF

THE GENUS EUCALYPTUS

The results embodied in this thesis represent my personal, original investigations with the exception of:

a. The electron microscope photographs of leaf surfaces

b. that portion of Section 5. 13 of Chapter V acknowledged therein to have been carried out in conjunction with Mr. R.J. Cameron.

These papers constitute a thesis submitted for the degree of Doctor of Philosophy in the Australian National University.

April, 1965.
ORIGINALLITY OF THESIS

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W.A. Heather
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Leaf diseases in the pathology of hardwood forests. The pathology of leaf diseases of hardwoods has received little attention. Discussion of such diseases, in texts on forest pathology, usually amounts to little more than noting the occurrence of the pathogen on the particular host and a brief statement indicating that the disease is unimportant (Peace, 1962; Boyce, 1961; Baxter, 1952). Lists of fungal leaf pathogens give taxonomic detail with little indication of the significance of their parasitism (Spaulding, 1956, 1958, 1961; Grove, 1935). This treatment of foliage disease fungi of hardwoods is in contrast with the pathological studies on the leaf pathogens of fruit trees (Butler and Jones, 1949) and to a lesser degree to that accorded to the leaf disease fungi of conifers (Boyce, 1961; Baxter, 1952).

It has been pointed out that hardwoods have good recuperative powers and are not seriously affected by foliage diseases (Boyce, 1961). However, photosynthesis is probably the physiological process of most importance to the forester and the one over which he has exercise some control (Richardson, 1960). It has been noted that if foliage infection is severe and continuous, the一圈 of growth diseases result in a general unhealthy condition, which predisposes the host to attack by other pathogens. This is a factor generally necessary to bring about the death of hardwood trees.
diseases (Boyce, 1961). However photosynthesis is probably
the physiological process of most importance to the forester
and the one over which he can exercise some control (Richardson,
1960). It has been noted that if foliage infection is severe
and continuous over a period of years, it must be reflected in
the reduced growth rate of trees. Such diseases result in
a general unhealthy condition, which predisposes the host to
attacks by other fungi and insects and lessens their resistance
to unfavourable weather conditions (Boyce, 1961).
Defoliation in successive years is generally necessary to bring
about the death of trees.
Foliation There are reasons, historical, geographical and stemming
from past forest management practice which partly account for
the present attitude to the fungal leaf pathogens of hardwoods.
More intensive utilisation and improved technology are resulting
in more interest in wood production from hardwood forests
throughout the world. The techniques of forest management
and silviculture result in reduced genetic variability in
forest crops. The dangers from disease, inherent in the reduced
genetic variability in such crops, have been stressed
(McNew, 1960; Heather, 1961a). It is significant to note in
this regard that the leaf diseases of poplar have received
increasing attention in forest pathology (Schreiner, 1937; guarded
Waterman, 1954). The tendency in the past has been to measure,
disease significance by its effect on the in situ forest, ignoring the importance in obtaining adequate regeneration for future management. This attitude has disregarded the concept of "disease impact" (United States Dept. of Agric., 1958) and has been adequately dealt with by Starr Chester (1959) in relation to agricultural crops. Intensive forest management of the future will be aiming at maximum wood production, consistent with the maintenance of other factors of the environment, from evergreen hardwood forests. It is likely that in the future foliage diseases of hardwoods will receive increasing attention.

Foliage diseases of the genus Eucalyptus are reported to be as considerable differences have been noted in the appearance of eucalypt foliage overseas by comparison with that of the same species in Australia (Penfold and Willis, 1961). Generally eucalypts grow more slowly within Australia than outside this country and the slower growth rate in their native land has been largely attributed to foliage damage (Jacobs, 1955). The limited life of eucalypt leaves apparently in Australia (average of eighteen months), compared with needles of evergreen conifers overseas (5-8 years), has been noted and (Jacobs, 1955). Responsibility for eucalypt foliage damage in Australia is mainly attributed to insects, fungi are regarded as being of less significance (Penfold and Willis, 1961; Jacobs, 1955).
There is a considerable amount of taxonomic data on fungi occurring on eucalypt leaves (Spaulding, 1958, 1961; Hedgecock, 1926; Cooke, 1892). There is very little information on the pathology of these organisms (Heather, 1961).

Some potentially important foliage diseases of Eucalyptus have already been recorded. *Puccinia psidii* has been recorded on *E. melliodora* in Brazil (Joffily, 1944; Da Silva, 1939). Powdery mildew has been noted on eucalypts in Italy and Great Britain (Crasso, 1948; Glasscock and Rosser, 1958). Mildew is of extensive occurrence and considerable significance on dry country species, when grown under glasshouse conditions, in Australia. The ash group of the eucalypts are reported to be susceptible to a rust coloured spot caused by an unidentified fungus in South Africa (Poynton, 1955).

**Aims of the present investigation**

In view of the above, it seems likely that fungal pathogens of eucalypt leaves will become more significant in the future.

The present investigation aimed at selecting an apparently active fungal leaf pathogen of eucalypt leaves and studying:

1. The factors of environment affecting its growth and pathogenicity.
2. Infection of the host leaves as a means of appreciating the epidemiology of the disease.
The histology and physiology of the host pathogen relationship. Such studies being basic to understanding the nature of resistance to the disease.

Phaeoseptoria eucalypti was selected as the pathogen for study. This organism has a wide field occurrence on commercially important species of the Macranthera subgenus of the eucalypts. It has been noted as an active parasite of eucalypt seedlings in the field and in nursery beds. Preliminary investigations showed that it was relatively easy to secure successful artificial inoculations of host species and that the organism could be cultured on artificial media.

The genus Phaeoseptoria does not appear to have been recorded elsewhere as a leaf pathogen of trees, but has wide occurrence as a parasite on grasses in the Western hemisphere (Sprague, 1943). The genus Septoria is of wide occurrence as a leaf and stem parasite of forest trees, ornamental shrubs, fruit trees, vegetable, forage and grain crops.

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CHAPTER I

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Walker observed that other academic staff of the Botany Dept, A.N.U., and fellow research students who have contributed by useful discussion and advice, and the typical conidiophores occurring in Dr C. J. Shepherd, C.S.I.R.O., Division of Plant Industry, for reading and criticising the original draft of the thesis.

I also wish to acknowledge my indebtedness to the Commonwealth Public Service Board, who granted me a two year scholarship to carry out this investigation and to the Forestry and Timber Bureau for their co-operation, in some P. eucalypti spores, (Plate 1/1). It should be noted that reproductions of illustrations of Phaeoseptoria festucae var
CHAPTER I


1.1 Taxonomy

Phaeoseptoria eucalypti was first described by Hansford (1957). Walker (1962) re-examined the type material in conjunction with additional samples of the organism and published an amended description. This dealt mainly with spore length, degree of septation, roughness of the spore wall and the nature of the conidiophores. Walker observed that the latter were of the type described by Hughes (1953) as annellophores. The distinction which Hughes has made between annellophores and the typical conidiophores occurring in pycnidia is that in the former the conidia are extruded successively from the tip through the series of concentric, annular scars left by previously detached conidia.

Observation of the material available confirms Walker's amendments. The frilled type of base characteristic of spores produced on annellophores is particularly obvious in some P. eucalypti spores, (Plate 1/1). It should be noted that reproductions of illustrations of Phaeoseptoria festucae var
PLATE 1/1

Septate spore of *Phaeoseptoria eucalypti*. Note the frilled type base characteristic of spores produced from an annellophore.
muhlenber giae (Sprague, 1943) also shows the frilled type base on spores of that species.

Walker (1962) has pointed out that certain workers regard the genus Phaeoseptoria Speg. as a synonym of Hendersonia Sacc. (Petrak, 1941). The distinction between the two genera is based essentially on the difference between fusoid (Hendersonia) and filiform (Phaeoseptoria) spores and does seem rather indefinite. (Barnett, 1955).

Forest Immature spores of Phaeoseptoria eucalypti are frequently less than 12 μ long and 3-5 μ wide with 0-2 septa. Such spores could be classified as Hendersonia SP. The mature spores, up to 70 μ in length x 3-5 μ with 3-5 septa, are typically filiform and characteristic of the genus Phaeoseptoria Speg. Walker has left the species in the genus Phaeoseptoria until further investigations reveal the relationship of the two genera.

1.2 Host Range.

The organism was originally recorded as a parasite on green leaves of E. grandis*. In the amended description (Walker, 1962) it was recorded on E. saligna from Canberra, E. pilularis E. grandis

* The authority for all Eucalypt species names used in this thesis is F. Blakley, 1955 "A Key to the Eucalypts" Forestry and Timber Bureau Canberra.
E. macarthuri, E. maculata and E. sideroxylon from West
Pennant Hills.

The following occurrences of the organism have been
noted during this study.

Field Records.
Canberra
Coffs Harbour
Pine Ck., State Forest
Sth., Q'land
Glass House and Nursery Records Canberra.

Artificial inoculation with heavy spore suspensions has shown that cer-
tain numbers of the Renanthera, Corymbosa and the box E. galatea
E. tereticornis E. macarthuri x E. viminalis hybrid
differences between spore germination on certain field sus-
ceptible species of the Macranthera and that on apparently field
resistant members of other sub genera (see Chapter 3) may
possibly explain in part the limited host range observed in
the field. Only light infections were induced by artificial
E. macarthurh
inoculation in E. maculata, E. melliodora and the Renanthera.
Records following artificial inoculations with heavy spore
susmissions. The only severe artificial infection achieved
E. pilularis E. grandis
E. fastigata E. saligna
E. melliodora E. grandis x E. pulverulenta hybrids
E. maculata
E. pulverulenta
27°C, nyctothermalture 22°C, Fluorescent lighting 8 hours per
day E. bicostata
In the field the organism appears to have a limited host range being recorded mainly on the Eastern Blue Gum Group and E. tereticornis of the Red Gum Group and in plantations of E. bicostata, all of the sub genus Macranthera, (Pryor, 1959). Under glasshouse and nursery conditions the organism shows a wider host range within the Macranthera and also occurs on the Corymbosa and an ironbark. Artificial inoculation with heavy spore suspensions has shown that certain numbers of the Renanthera, Corymbosa and the box E. melliodora are susceptible. The quantitative and qualitative differences between spore germination on certain field susceptible species of the Macranthera and that on apparently field resistant members of other sub genera (see Chapter 3) may possibly explain in part the limited host range observed in the field. Only light infections were induced by artificial inoculation in E. maculata, E. melliodora and the Renantherous species. The only severe artificial infection achieved on species outside the Macranthera was on a particular plant of E. fastigata. This particular plant after inoculation and incubation was kept in the L.B. Growth Cabinet (Pescod, Read and Cunliffe, 1962) until symptoms of the disease developed. The particular environmental conditions (phototemperature 27°C, nycotemperature 22°C, fluorescent lighting 8 hours, ...
photo period (lights 8 hours and 8 hours darkness) may have been important factors in this instance. Artificial inoculations revealed no completely resistant species. On the latter plates of plates of white light agar and water agar containing discs of P. eucalypti was isolated from diseased tissue of E. bicostata, E. tereticornis and E. grandis. Successful isolations from diseased tissue could be obtained at any stage from the time of symptom production, until the infected area was completely necrotic and the organism was fruiting profusely. It was found that the most successful isolations were from tissue at an early stage of symptom development, i.e., when the leaves showed a slight chlorotic flecking. Isolations from such tissue were usually pure if the surface sterilisation was carefully carried out. Isolations from tissue at more advanced stages of disease development were frequently contaminated, presumably by organisms which had invaded the necrosed area of the leaf. A more fusoid (30-40 μ) Pure cultures were also obtained by plating whole inoculum spore horns pricked off individually from the diseased leaf. Single spore cultures were obtained readily by taking leaves which had just commenced to show spore horn production and brushing the horns into sterile water with a heat sterilised camel hair brush. This spore solution was then poured
onto the surface of agar plates and single isolated spores selected, after some 48 hours, when germination was obvious.

*P. eucalypti* was successfully cultured on sterile malt agar, malt vegemite agar and water agar containing discs of susceptible host tissue as nutrient. On the latter the colonies reached a diameter of 0.7 cm in 3 weeks and 1.2 cm in 6 weeks.

The colonies were initially hyaline and darkened to an olive brown after 2-5 days. Under the microscope the hyphae were lightly branched and contorted. The final colonies produced showed a white superficial mycelia which was very thin and powder like. Underneath the colony was greenish black and examination of this sub surface growth showed that it was made up of pycnidia of the fungus set in a mass of olive brown hyphae. The pycnidia sporulated at 15-17 days from a single spore culture. The spores were essentially similar to those on the host leaves being olivaceous and 3-5 septate. They were generally somewhat more fusoid (30-40 μ long and 3.5-5 μ wide) than the characteristic filiform spores produced on the host. The underside of the culture was black. The growth on media was almost completely superficial, penetrating only 1-2 mm. In culture as in the living host (Plate No. 1/2) the hyphae showed frequent right angle bends. Where these occurred chlamydospore like body was
Intercellular hyphae within the mesophyll tissue of *E. bicostata* leaf. Note angular bends in hyphae and production of chlamdospore like body at bend in high power photograph.
formed. This body was spherical, highly protoplasmic and had a thicker wall than the hyphae. It appeared to be firmly attached to its parent hyphae and its significance is not evident. These bodies have been noted elsewhere in the genus Septoria (Heather, 1961).

1.4 Proof of Pathogenicity

Koch's postulates for proof of pathogenicity were satisfied on both E. tereticornis and E. bicostata. The organism was isolated separately from both host species when the leaves were at the stage of mild chlorotic flecking. 2.5 cm diam. pieces of leaf tissue containing infected and apparently uninfected leaf tissue, were surface sterilised in 1% Silver Nitrate for 3 mins, excess sterilant precipitated with a saturated solution of Sodium Chloride. The pieces were then plated on malt-vegetable agar. Plants of E. bicostata and E. tereticornis were inoculated with cultures obtained from both of the original host plants. Host leaves were sprayed with a culture suspension containing spores and mycelia. The plants were incubated for 48 hours at 25°C in a humid environment with additional light from 2 Condor lamps (150-watt tungsten filament internal reflector incandescent lamps giving an incident light flux of .65 ft. candles). After 30-35 days symptoms typical of the disease developed and P. eucalypti pycnidia sporulated on the diseased areas.
after 40 days. At 30 days after inoculation reisolation of the pathogen was made from inoculated leaves which showed light flecking. The characteristics of the two cultural isolates were identical. They corresponded well with the descriptions of *P. eucalypti* (Hans.) Walker, differing only in the more fusoid shape of their spores which fell towards one extreme of Walker's (1962) description. *P. eucalypti* This test established *P. eucalypti* as the casual organism of the disease. It also indicated that isolations from *E. bicostata* and *E. tereticornis* were pathogenic on each of the host species selected. There was no attempt to evaluate the relative pathogenicity of each isolate to each host species. The gas exchange measurements, respiration and gas exchange measurements.

1.5 **Histology of Infection and Host Pathogen reactions.**


A study was carried out of the process of infection and the histology of host pathogen reactions on infected leaves. The leaves used for this study, were the lower juvenile leaves of the six *E. bicostata* plants used in the gas exchange experiments (see Chapters 7 and 8). The plants were raised from seed germinated in the Ceres phytotron. At 10 days, the cotyledonary stage seedlings were pricked out into 5" pots of perlite, 3 seedlings per pot. The plants
were transferred to a glasshouse in the phytotron, photo-temperature 27°C, nycotemperature 22°C, watered with half strength Hoagland's solution in the mornings and demineralised water in the afternoons. At 3 weeks the seedlings were reduced to 1 per pot. At 12 weeks the lower 4 pairs of leaves, with the exception of one leaf on each plant, were inoculated with a spore suspension of *P. eucalypti*. The inoculation was made with a heat sterilised camel hair brush on the undersides of the leaves. The juvenile leaves of *E. bicostata* are hypostomatous and preliminary investigations indicated that invasion usually occurred through stomata. The leaf left uninoculated was to be used as the "control" in the gas exchange measurements. The inoculated plants were incubated for 4 days at 25°C in a high humidity cabinet with additional light from two Condor Lamps. They were then transferred to the L.B. growth cabinet and arranged for gas exchange measurements (see Chapter 6). The lower leaves, not being used for these measurements, were sampled at 2 day intervals for a study of the infection process and the histology of host pathogen relationships. The sampling was normally made after the 8 hours fluorescent light period in the L.B. cabinet.

Pieces of infected leaf 3.5 cm square were cut out and placed immediately in 95% Methanol for 4-8 hours to remove
the chlorophyll. The samples assumed an overall pearly appearance. After this treatment, the leaf pieces were halved. One half was placed in clear lactophenol, the other half in distilled water. In about 1 hour that in lactophenol was completely translucent. It was taken and gently simmered in Lactophenol Cotton Blue for 3 mins and allowed to cool (White and Baker, 1954) and then mounted underside uppermost, on clean slides in clear lactophenol.

The leaf piece placed in distilled water was usually left for some 24 hours to rehydrate, then placed in Lugol's iodine and gently warmed. After cooling they were mounted underside uppermost in 10% glycerine. The purpose of this iodine staining was to indicate starch production and accumulation.

A summary of the histology of infection is presented below. The details of these observations, in chronological order are presented in Appendix 1. The histology of host-pathogen reactions was studied on permanent sections of infected juvenile leaves of E. bicostata fixed in formaldehyde-acetic acid-alcohol, embedded by a modified Johansen schedule and stained in safranin-light green combination.

Summary of histological observations

At seven days after inoculation the spores were swollen and many had germinated usually forming two germ tubes from
the terminal cells of the spore penetration was exclusively stomatal (Plate 1/3). An appressorium was produced on the stomatal slit like pore (Plate 1/4). The germ tubes appeared to spread at random on the leaf surface with no evidence of a stimulus directing germ tube growth towards stomatal pores (Plate 1/5). Actual penetration was frequently by a branch hypha (Plate 1/6). The tip of the main germ tube continued growth and on occasions penetrated other stomata. At two weeks there was extensive spread of intercellular hyphae from the sub stomatal cavity and permeating the spongy mesophyll tissue. At seventeen days after inoculation there was conspicuous bunching of hyaline hyphae in the cavities of stomata adjoining the stoma originally penetrated. At this stage there were no microscopic symptoms of the disease and the chloroplasts, even those in guard cells of penetrated stomata, appeared normal. Sugar to starch conversion appeared to be occurring in the invaded areas.

At twenty three days the leaf pieces failed to clear completely in the 95% methanol (Plate 1/8). Small "green areas" retained their colout, these were extensively permeated by inter cellular hyphae. The structure of the chloroplasts in the spongy mesophyll in these areas was destroyed. These "green areas" continued to enlarge until they reached the veins surrounding the infected islet. Sugar to starch conversion
PLATE 1/3

Stomatal penetration of leaves of *E. bicostata* by germ tubes of *P. eucalypti*.

Top photograph in face view, lower photograph shows in section the hypha passing between the guard cells of the stoma.
Appressorium on the lips of the guard cells of a stoma in a leaf of *E. bicostata*. The appressorium is below the overlying cuticle.
PLATE 1/5

Apparent random spread of germ tubes over the surface of E. bicostata leaf.
PLATE 1/6

Stomatal penetration of leaf of *E. bicostata* by side branch of main germ tube from a *P. eucalypti* spore.
"Green areas" produced on infected leaf of *E. bicos­tata* 23 days after inoculation with *P. eucalypti*. Note the general angular outline of the spots.
At twenty six days microscopically apparent aleurone spots develop on the leaves. These correspond to the "pale-tissue" described above and were generally observed as clusters of small clear spots. The spots were brown bunched together in a pattern (Pl. X, Fig. 3). The aleurone cells are often elongated and pointed on the tips. This elongation is particularly evident in the irregular spots.

3. Differentiation of aleurone tissue and starch particles

The appearance of the aleurone and starch particles, as described above, is in agreement with the observations of Shively (1938, 1943) who studied the leaf development in Avena sativa.
At twenty six days microscopically apparent chlorotic spots developed on the leaves. These corresponded to the "green areas" described above and had a generally angular outline. The bunches of hyphae in the substomatal cavities had enlarged and darkened to a deep brown colour. (Plate 1/9).

Sporulation commenced at thirty days on the chlorotic spots. The fructifications which had developed from the brown bunched hyphae were located immediately below the stomata (Plate 1/10). The spores were exuded as a horn through the stomatal pore (Plate 1/11). The chloroplasts of the guard cells over the fructifications appeared normal although those in the adjoining spongy mesophyll had lost their usual structure.

Final observations at thirty five days showed heavy sporulation with horns of spores protruding through stomatal pores. The infections were restricted by veins and necrosis was evident in these angular spots.

1.5.3 Discussion of histology of infection and host pathogen reactions.

The apparent random spread of germ tubes, some passing beside and even over stomata before penetrating a more distant stoma, is in agreement with the observations of Septoria passerinii on barley (Green and Dickson, 1957). It is in
Bunched hyphae of *P. eucalypti* in the substomatal cavities on the abaxial surface of a leaf of *E. bicostata*. These bunched hyphae develop into pycnidia of the fungus (see plate following).
PLATE 1/9 (cont'd)

Dark fructifications in the substomatal cavities.
PLATE 1/10

Pycnidium of P. eucalypti with the ostide corresponding to the position of the stomatal pore.
Exudation of spores of *P. eucalypti* through the stomatal pore on the abaxial surface of a leaf of *E. bicos-tata*.
contrast with observations on certain other organisms where there are indications of a stimulus directing hyphal tip growth towards stomata (Bald, 1952; Dickinson, 1949). Penetration of stomata by side branches of the main germ tube has been recorded with Pellicularia filamentosa from the (Flentje, 1959). A single germ tube as a consequence never frequently penetrated several stomata before tip growth mainly ceased in the mesophyll spaces, between mesophyll cells and to Eucalyptus appears to be an obligate stomatal penetrated etrant. Swellings in germ tubes, interpreted as attempts at direct penetration, all appeared to be abortive. This is in general agreement with the observations of Green and Dickson (1957). In certain rusts penetration has been observed to be either direct or stomatal (Allen, 1922). Stomatal appressoria produced on the lips of the guard cells under the overlying cuticle of the stoma has been recorded elsewhere (Allen, 1923; Pristow and Callegly, 1954). Green and Dickson (1957) record a thickening of the germ tube of S. passerini before passing between the guard cells. It has been suggested that stomatal penetration is probably forced against the narrow opening of the guard cells (Flentje, 1959). It was noted that in rare instances where two germ tubes entered the same stoma, both produced appressoria. This suggests that the resistance of the guard cells to separation may not be the sole factor...
stimulating appressorial production of leaf spots on dicots. The spread of intercellular hyphae without the development of haustoria within the leaf of *F. bicostata* agrees very well with that given by Green and Dickson (1957) for *S. passcerinii* on barley. The hyphae spread quickly from the penetrated stoma to the cavities of adjoining stomata never penetrating between epidermal cells. The hyphae spread mainly through the mesophyll spaces, between mesophyll cells and to a limited degree in the palisade tissue. Hyphal spread halted at the vascular bundles as has been observed by Green and Dickson (1957) with barley leaf blotch, *sce in the stem*.

(Hursh The rapid, presumably parasitic, spread in living tissue following penetration distinguishes this organism from a number of other fungal parasites of *Eucalyptus* leaves which behave as latent infections e.g., *Gumannardia citri-campe* (Kiely, 1948). The spore of *P. eucalypti* germinates and grows readily on artificial media, its germ tubes carry out parasitic spread following stomatal penetration without perceptible pause. This combination of saprophytic and parasitic ability suggests that the enzyme equipment of the spore must be a relatively complete one (Gottlieb, 1964).

The limitation of individual infections by veins surrounding islets is in contrast with certain other organisms which invade and spread past veins e.g., *Peronospora tabacina*.
It has been observed that leaf spots on dicotyledonous plants are sometimes angular. Lack of penetrative force or the absence of an effective cellulytic or lignolytic enzyme system have been advanced as possible reasons for this (Dickinson, 1960). The same feature of limitation of infection by mechanical tissue has been observed in the monocotyledons. Eutyloma oryzae the causal organism of rice leaf smut is frequently limited by vein boundaries (Shimada, 1957). In certain wheat varieties the degree of stem infection by Puccinia graminis triticii is related to the degree of mechanical tissue in the stem (Hursh, 1924).

It has been noted that in rice the silicic acid content of the leaves is frequently inversely related to disease incidence (Akai, 1959). The veins of E. bicostata and a number of other eucalypts have extensive deposits of silica in the superficial cells surrounding their veins and in apparently randomly distributed cells elsewhere (Plate No. 1/12). It seems likely that the sclerenchyma like cells surrounding the vascular tissue of the veins and the high silica concentrations in these areas may both contribute to limiting lateral spread of the pathogen within the leaf.

The production of fructifications in the substomatal caustics is of considerable interest. Green and Dickson
Silica in the cells along vein margins and in randomly distributed cells elsewhere in the leaf of *E. bicostata*.

(See continuation of plate 12 also).
PLATE 1/12 (cont'd)

Silica in marginal cells surrounding veins of
E. bicostata leaf. Note also the sclerenchymatous bundles
surrounding the vascular tissue.
record that \textit{S. passerinii} produces its fruits under the stomata usually on the upper surface of barley leaves. The juvenile leaves of \textit{E. bicostata} are hypostomatous and in these the pycnidia are produced exclusively on the lower surface. In intermediate and leaves of \textit{E. tereticornis} fructifications are produced on both surfaces. The fructifying of fungi through stomatal pores has been recorded e.g. \textit{Phytophthora infestans} (Berkley, 1846) but little attention has been paid to this aspect of fungal behaviour. On occasions the fructifications of \textit{P. eucalypti} appear to shed their spores inside the host leaf (Plate No. 1/13). It is possible this rarely observed phenomenon occurs where the ostiole of the pycnidium does not coincide with a stomatal pore.

The stomata are obvious places of discontinuity in the leaf surface. An organism which has the capacity to produce its fruit bodies in or adjacent to stomatal cavities, so that the spores can be shed without the necessity of rupturing the epidermis and cuticle would appear to have an advantage of distinct value. This advantageous position must surely be achieved by reaction to a physiological stimulus. The physical forces which might tend to restrict fructification development and spore exudation would not act on the fruit body until after the fruit primodia were formed.

The gradients which must exist in the concentration of
Pycnidium of *P. eucalypti* shedding spores inside the leaf of *E. bicostata*. This may occur when the ostiole of the pycnidium does not correspond with the stomatal pore.
ases or other materials between the stomata and the mesophyll cells could be the physiological factors responsible for the reaction. Muller's (1959) classification of E. eucalypti on E. bicolor is at least a transitional pathogen combination. The process of parasitic spread of the intercellular hyphae through living tissue following penetration. There was a period of at least 16 days between penetration and the death of infected tissue. Presumably during this period the organism was living parasitically on the host cells. This is a highly significant observation for it places the organism in the category of a biotroph (White, 1957) at least so far as its parasitic spread is concerned. Muller (1959) in a review of hypersensitivity has indicated that in the case of true necrotrophic fungi no eusymbiotic relationship even a temporary one seems to occur. In the case of the biotrophic fungi "a high degree of mutual tolerance between host and pathogen in the presence of susceptibility is characteristic." (Muller, 1959). This author considers the host-pathogen combination of Pyrus malus and Venturia inaequalis a transition type between typical biotrophs and necrotrophs. He has included the occurrence of S. passerinii on barley as an instance where no eusymbiotic relationship seems to occur. It is impossible to assess from the paper of Green and Dickson (1957), the period during which the cells of susceptible
variety barley leaves remained alive following the penetration of the leaf.

On Muller's (1959) classification \textit{P. eucalypti} on \textit{E. bicostata} should be regarded as at least a transitional type and possibly as a facultative biotrophic combination. If a host pathogen combination could be found which illustrated hypersensitivity leading to resistance the classification of host pathogen complex would be clearer.

It was observed that necrosis occurred first in the mesophyll cells (Plate No. 1/14) this is in agreement with work of Green and Dickson (1957) on \textit{S. passerinii}. It was also noted that as the disease progressed the nuclei of the host cells stained deeply with safranin in the safranin light green stain combination. This also agrees with the observations of Green and Dickson (1957).

There was no evidence of any histological barrier to infection such as that described for certain host pathogen combinations in \textit{Eucalyptus} leaves (Heather, 1961) and considered as a common reaction type in diseased leaves (Cunningham, 1928). This is in agreement with the observations on leaf spots caused by \textit{Septoria sp.} (White, 1957).

1.6 Conclusions

1. The taxonomic description of \textit{P. eucalypti}, as amended by Walker, has been confirmed. The difficulty of dist-
Necrosis in the cells of the mesophyll tissue of a leaf of *E. bicostata* infected with *P. eucalypti*.
1. Artifical inoculations have shown that no Eucalyptus species tested, shows complete resistance.

2. It has been established that *P. eucalypti* (Hans.) Walker is the causal organism of Phaeoseptoria leaf spot of eucalyptus.

3. Infection is exclusively stomatal. Attempts at direct penetration observed have not been successful.

4. The organism produces a small appressorium, below the overlying cuticle and over the stomatal pore. After penetration, *P. eucalypti* spreads intercellularly through living host cells.

5. Host cells in the invaded area remain capable of starch synthesis and presumably alive for some 16 days after invasion. Chloroplasts of the guard cells of invaded stomata do not become disorganised.

6. The organism only spreads as far as the vein boundaries which apparently provide a mechanical or chemical barrier to further penetration.

7. Pycnidia of *P. eucalypti* are produced in the substomatal cavities of stomata adjoining the penetrated stoma, and spore cirri are exuded through the stomatal pore.
Spore Germination in relation to the Physical Factors of the Environment

Spore germination is the transformation from a dormant state of low metabolic activity, to one of high activity (Gottlieb, 1964). This is also a fundamental problem of biological interest.

Consideration of spore germination of the pathogen is consequently of significance in the study of the ecology and pathology of particular plant diseases. The study of spore germination has concentrated on three aspects -

1. Spore germination on detached leaves of Eucalyptus
2. Spore germination in relation to the physical factors of the environment
3. Spore germination to leaf exudates

For many fungi, the spore is the sole means of dissemination and survival in critical periods. The germination of spores for these fungi is an essential part of their life cycle. Prevention of germination of spores of plant pathogenic fungi, as a means of disease control, has always been attractive to plant pathologists.

Introduction

For many fungi, the spore is the sole means of dissemination and survival in critical periods. The germination of spores for these fungi is an essential part of their life cycle. Prevention of germination of spores of plant pathogenic fungi, as a means of disease control, has always been attractive to plant pathologists. Germination, in its essence, the transformation from a dormant state, of low metabolic activity, to one of high activity (Gottlieb, 1964), is also a fundamental problem of biological interest.
Spore germination in relation to leaf exudates from a known host - *E. bicostata*.

It has been pointed out that the formation of germ tubes by fungal spores is evidence that the physiological processes leading to germination are complete, i.e., that the spore has been transformed from a dormant to an active state (Gottlieb, 1964). The various methods used by workers to measure germination have been reviewed and discussed (Cochrane, 1958; Hawker, 1950). In this study, two methods have been used.

2.2 Assessment of Germination

**Method 1:** Number of Germ tubes per group of 25 spores. Basically, this method is equivalent to Cochrane’s (1958) "fraction of spores that in a given time forms a germ tube". The only modification is that the total number of germ tubes produced by the spores was counted rather than scoring for the presence of a germ tube alone. This modification was desirable because of the multicelled nature of the spore and consequently the frequent production of several germ tubes by a single spore. It was reasoned that with a spore whose germ tubes penetrated by stomata but showed no apparent tropic response to the position of the stomata, the production of numerous germ tubes increased the probability...
of infection. 

This is not necessarily always the case since in vitro a spore producing numerous germ tubes generally had shorter tubes than a spore producing only one. It seemed a reasonable compromise however to allow for the increased inoculum potential of a spore producing several germ tubes. Hereafter this method is referred to as "the number of germ tubes in 25 spore groups". It was only used when the evident variability of germination in a treatment was low. In assessing germination for any treatment at least 8 groups of 25 spores were scored as and Methods for "in vitro" germination.

Method 2: Preliminary investigations showed a high variability in successive counts of "germination" as measured by the above method and still more so if the simpler method of merely counting the number of spores which formed a germ tube in a given time were used. The relatively long period necessary to achieve germination of all viable spores and the multicelled nature of the spore probably both contributed to this variability. It was found that a system of scoring which took into consideration recognisable stages in spore morphology during germination as well as germ tube formation reduced this variability. In this method of scoring, weight was also given to the number of germ tubes produced for the reasons outlined in Method 1 above.
The criteria used in this scoring method are illustrated in Figure 2/1.

In this scoring procedure once again the spores were scored in groups of 25. At least eight replications of these 25 spore groups were scored for each treatment in the following experiments. Hereafter this method is referred to as "the ontogenetic scoring method". The score is given as "the mean ontogenetic germination score for 25 spores". This is the mean of at least eight replications and the standard error of this mean was computed from these replications.

2.3 Materials and Methods for "in vitro" germination experiments

The methods used have been in general the same throughout. These are given in detail in Appendix 2 and summarised below.

The germinations were carried out in crystal glass staining dishes. When containing 6 ml of germination medium they had a width of surface exposure three times the depth of the medium. These dishes were placed in covered petri dishes whose bases were overlain with wet vermiculite and filter paper. The spore solutions were obtained by brushing horns from infected leaves. After collection the spores were washed three times in distilled water using a centrifuge. The spores were stored in a cold room at 1^\circ-3^\circC.
FIGURE 2/1

Diagrammatic illustration of criteria used in scoring by the ontogenetic scoring method. Stages 1 - 5 are commonly observed in the germination of single spore. 6a and 6b indicate the method of scoring a spore which shows a more complex germination pattern.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aseptate spore</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Septate spore</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Septate swollen spore</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Septate swollen spore with one germ tube</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>Septate swollen spore with two germ tubes or two tips on one germ tube</td>
<td>4</td>
</tr>
<tr>
<td>6a</td>
<td>Septate swollen spore with four germ tube tips</td>
<td>6</td>
</tr>
<tr>
<td>6b</td>
<td>Septate swollen spore with four germ tubes</td>
<td>6</td>
</tr>
</tbody>
</table>
The typical layout of dishes for an experiment is given in Figure 2/10. The dishes were incubated in the dark at 25°C for 48 hours.

At the conclusion of the germination period the treatments were sampled, two aliquots taken from each and placed on separate cleaned glass slides. The spores were dried on the slide, and stained with Lacto-phenol-cotton blue. The germination of 4 groups of 25 spores was assessed for each slide.

The results were analysed by computing the mean and standard error of the mean for at least eight replicates of 25 spores per treatment. The results were plotted in a series of graphs or given in tables. Where necessary student's T test was used to assess the significance of the difference between means of treatments. The value of \( P = 0.05 \) was taken as an acceptable level for significance of differences.

2.4 Preliminary Experiment No. 1 Germination of spores in spore solution.

This was a preliminary experiment to see how the spores germinated in relation to time and other factors. In this instance the spore solution was collected from infected leaves and diluted with distilled water until a haemocytometer count gave a concentration of 32,000 per cc. To 0.4 ml of this spore solution, 1 ml of Citric Acid Phosphate Buffer pH
FIGURE 2/2a

Diagrammatic illustration of the layout of staining dishes in a typical in vitro germination experiment.
4.0 was added in a crystal glass staining dish. The dishes were placed in an incubator at 25°C in the dark, examined at regular intervals over the next 48 hours by which time no further germination appeared to occur. The germination solutions were then sampled and assessed using the ontogenetic scoring method. Germination in spore solutions has been usually recorded (Krishnan, Bajaj and Damie, 1954; Keitt, 1959). Mean ontogenetic germination score of 25 spores is 26.0 + 32.0. The minimum possible score for 25 mature (i.e. septate) spores would be 25, this score is obviously very low. On average, only one spore in 25 had advanced past the septate stage after 48 hours. Several possible explanations suggested themselves. Age of essential materials from the spore is likely to be such low germinations are characteristic of this organism. A likely cause could be broken physical or chemical inhibition by either physical or chemical treatment. From the first centrifuging (spore washing) the water treatment was not the optimum for germination e.g. temperature.
2.5 Investigation of possible presence of an inhibitor of germination in spore solutions.

The aim of the experiment was to try to eliminate possibility i.e. the presence of an inhibitor. The presence of inhibitors of spore germination in spore solutions has been frequently recorded. (Krishnan, Bajaj and Damle, 1954; Keitt, Blodgett, Wilson and Magie, 1937). The washing of spores collected in crude spore solutions to eliminate the possible presence of such inhibitors before using the spores in germination experiments is a common procedure (Shepherd, 1962). The major objection to such washing of spores is that, in the case of spores with very thin walls being washed in distilled water, leakage of essential materials from the spore is likely to occur.

2.5.1 Experiment No. 2 Germination of unwashed spores in aliquots of spore washings in distilled water.

The crude spore solution collected from infected leaves adjusted to a spore concentration of 32,000 per ml was divided into two halves and one half stored. The other half was washed according to the procedure outlined (Appendix 2). The water collected from the first centrifuging (spore washings) was kept and used in the germination media in this experiment.
1 ml Citric Acid/Phosphate buffer pH 6.0 and 1 ml of unwashed spore solution were placed in each of 6 staining dishes. The appropriate germination media was added to each dish. The germination media added were as follows:

<table>
<thead>
<tr>
<th>Dish No.</th>
<th>ml of distilled water</th>
<th>ml of Spore washings</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The dishes were incubated, sampled and assessed for ontogenetic germination score.

The results are given in tabular form in Appendix 2 and plotted in Fig. 2/2.

The results obtained show that dilution of the unwashed spore suspension by distilled water increased the germination of unwashed spores. This suggests that an inhibitor of spore germination was present in the unwashed spore solution and that the effect of this inhibitor was reduced by dilution.

To confirm this result a series of aliquots of distilled water plus the solution obtained by centrifuging unwashed spores was used as germination media for washed spores.

Comparison of germinations of washed and unwashed spores
Experiment 3  Germination of washed spores in aliquots of spore washings in distilled water

The same methods of setting up, incubation and assessment were used as those described in Expt. 2, except that in this instance, 1 ml of washed spore solution, concentration 32,000 per ml was added to the 0.1 ml of Citric Acid/Phosphate buffer pH 6.0 before adding the appropriate germination medium to each glass dish. The treatment media added were:

Dish No.  ml of distilled water  ml of Spore washings
1   0.5  0
2   0.4  0.1
3   0.3  0.2
4   0.2  0.3
5   0.1  0.4
6   0   0.5

The results are given in table form in Appendix 2 and plotted in Fig. 2/2.

2.5 3 Discussion Experiments 2 and 3

These results indicate that there is an inhibitor of spore germination present in the original spore suspension. The effects of this inhibitor can be reduced by dilution with distilled water or by centrifuging the spore solution and resuspending the spores in distilled water.

Comparison of germinations of washed and unwashed spores
Mean Ontogenetic Germination Score of washed and unwashed spores of *P. eucalypti* in media made up of Distilled Water and the solution centrifuged from washed spores at a series of concentrations.
Mean ontogenetic germination score for 25 spores

Percentage concentration distilled water in germination medium
in the two Experiments show that spores of the same origin have a lower germination (Sig @ P < .05) after centrifuging in media containing distilled water at a 33% concentration. The differences between the washed and unwashed spores in origin media containing distilled water at a concentration of 83%, 66.5%, 50%, and 16% are in the same direction although not due significant at P = .05 level. These results indicate that centrifuged washing of spores reduces their germination by comparison with non-centrifuged spores. Conditions (Allen, 1955). The reduction in germination following centrifuged washing is possibly due to physical damage. However, it has been shown that washed spores (by centrifuging) in the case of Peronospora tabacina have a Riboflavin requirement for germination while unwashed spores do not have such a requirement (Shepherd, 1962). The suggestion here is that thin-walled spores readily lose materials through their walls when they are washed. The thinness of the wall of Septoria apii has been emphasised and the difficulty in plasmolysing such spores indicates that their walls are readily permeable to a variety of materials (McMillan and Plunkett, 1942). It is likely that the wall of Phaeoseptoria eucalypti is similar and materials essential for germination may be leached during washing. The uneveness of the wall thickness has been mentioned in the amended taxonomic description.
(Walker, 1962). The thin areas noted may be places from which essential materials are lost.

These experiments while indicating the presence of an inhibitor in crude spore solutions do not indicate the origin of this material. In the case of *Puccinia graminis var. tritici*uredospores, self-inhibition has been shown to be due to a heat-stable substance—or substances—inactivated or removed from solution by glass surfaces. The material is a spore metabolite produced under aerobic conditions. (Allen, 1955). In the case of *Aspergillus niger* (Krishnan et al., 1954) and *Coccomyces haemalis* (Keitt et al., 1937) evidence suggests the material is present in the spore at the time of its formation. The presence of autoinhibitors of germination in freshly collected spore solutions of *Peronospora tabacina* has been demonstrated (Shepherd and Mandryk, 1962) and the isolation and properties of this material have been described (Shepherd and Mandryk, 1963). The possible ecological functions of such autoinhibitors have been discussed (Cochrane, 1958).

The methods used in some of these studies do not enable the distinction to be made between spore solutions in which an inhibitor is present produced by the spores themselves (auto-inhibitor *sensu strictu*) and spore solutions prepared by washing spores from leaves in which case the material...
could be either from the spores or from the infected leaf.

If infected leaf tissue produced the inhibitor then the material could be one which occurs also in uninfected leaves. (Chapter 4 shows that inhibitors of spore germination do occur in the water extracts of _E. bicostata_ leaves). Alternatively the material could result from the host pathogen reaction which has occurred in infected leaves. An inhibitor of either type which occurred at sufficient concentration on the surface of infected leaves could prevent the germination and consequent wastage of spores which landed on such tissue. Spores produced on such tissue would be available for spread by rain splash or rain wash from the leaf at a later stage. Adaptation by the funus to such an inhibitor would, from this viewpoint, have the same survival advantage as adaptation to an auto-inhibitor _sensu strictu_: On the other hand such an inhibitor might be quite ineffective in preventing germination in fructifications or in spore horns.

There is some evidence that in the case of _P. eucalypti_ the inhibitor is on the leaf surface rather than on the spores themselves. In spore horns pricked off individually and placed in drops of distilled water the individual spores germinate even if the horn does not disintegrate (Plate No. 2/1).

The question of the origin and nature of this inhibitor
PLATE 2/1

Germination of the spores of *P. eucalypti* in a detached, partly disintegrated spore horn in distilled water.
of germination could be profitably pursued further. This is particularly so since inhibitors investigated in detail elsewhere have been all essentially of autoinhibitor sensu strictu type. There is in literature abundant evidence of the presence of materials in uninfected hosts which have fungicidal possibilities (Kirkham, 1959) and also of fungicidal materials, apparently resulting from host pathogen reactions in infected hosts (Kiraly and Farkas, 1962). The general scheme of approach to the isolation of the inhibitory substance might follow that used in Chapter 4 for the isolation from E. bicostata leaves of promoters of germination. In all further "in vitro" germination experiments up for washed spore solutions were used. The remaining experiments concern the influence of other physical environmental factors on germination. The results of this experiment are given in tabular form.

**Preparation of a Time-Curve of Germination**

It can be expected that if the germination of spores in solution sampled at time intervals is plotted against time from commencement of the experiment (the so-called time-curve of germination) the general shape of the curve will be sigmoid (Cochrane, 1945). The degree to which the shape will be truly sigmoid will depend on the ratio of the time between successive samplings to the time for observation away. The lower this
ratio the closer will the curve approach the classical sigmoid shape. This difference is exemplified in Cochrane's data for *Phragmidium mucronatum* (Cochrane, 1945) and that of Wellman and McCallan for *Alternaria solani* (Wellman and McCallan, 1942). Preliminary observations had shown that *P. eucalypti* germinated over a long period. Thus with fairly long intervals between samples it seemed likely a sigmoid curve would be obtained.

12 Crystal Glass Dishes were taken and in each the following solution was placed: 1 ml washed spore solution concn. 32,000 per ml, 4 cc distilled water, 1 ml Potassium Hydrogen Phthalate buffer pH 4.0. The dishes were set up for germination as described previously, sampled at intervals and scored by the ontogenetic germination score method.

The results of this experiment are given in tabular form in Appendix 2 and plotted in Figure 2/3.

The resulting curve was typically sigmoidal. The germination reached a maximum at about 27 hours. The slight rises after this are not statistically significant.

The preparation of such a curve was important if experiments were to be conducted to compare the effects of various treatments on spore germination. In such treatment experiments it is essential to fix the time for observation away from the steep part of the curve, otherwise small time
Mean ontogenetic germination score for 25 spores

Time (days)
interval differences between the samplings could considerably affect assessment. Random distribution of forms from different treatments may affect the final maximum germination achieved and/or duration of germination throughout the experiment (Chapter 4, Appendix 4). Either of these effects individually may have considerable ecological significance. This is particularly the case with an organism where a reduction of one or more hours in the time taken to reach a particular germination level may be much more important for the future establishment of the organism than an increase of many per cent in the total germination achieved after a long time interval.

2. 7. Experiments on Spore Longevity and Method of Storage.

The period during which a detached spore remains viable and the conditions under which viability persists longest are of considerable ecological interest. In the case of pathogenic organisms which reproduce only by short-lived spores the absence of a suitable host for a brief period, or the onset of unfavourable conditions for spora survival, may cause a break in the pathogen's life cycle. From the leaves of E. bicostata carrying numerous recently produced spore horns were selected. The horns were dried, brushed onto a 20 cm disc of filter paper in a petri dish,
interval differences between the samplings could considerably affect assessment. Various treatments may affect the final maximum germination achieved and/or the rate of germination throughout the experiment (Chapter 4, Appendix 4). Either of these effects individually can have considerable ecological significance. This is particularly the case with an organism requiring a long period to achieve its maximum germination level. For example, a reduction of one or more hours in the time taken to reach a particular germination level may be much more important for the future establishment of the organism than an increase of many percent in the total germination achieved after a long time interval.

2.7 Experiment 5 Spore Longevity and Method of Storage

The period during which a detached spore remains viable and the conditions under which viability persists longest are of considerable ecological interest. In the case of pathogenic organisms which reproduce only by short-lived spores, the absence of a suitable host for a brief period, or the onset of unfavourable conditions for spore survival, may cause a break in the pathogen's life cycle. Leaves of *E. bicostata* carrying numerous recently produced spore horns were selected. The horns were dried at 0.5°C, brushed onto a 20 cm disc of filter paper in a petri dish.
The leaves were moved over the disc while being brushed to obtain a reasonably random distribution of horns from different parts of the same leaf and from different leaves, on the surface of the filter paper. The original filter-paper disc was then divided into quadrants, each of these was then redivided into 4 sectors with a sharp scalpel. 4 sectors (one from each of the original quadrants) were then used in each of the following 4 treatments.

A. The horns were brushed into 0.5 cc distilled water. This spore solution was stored in a refrigerated room at 0.5° - 3.5° C.

B. The horns were brushed onto a 9 cm filter paper disc and stored dry in the refrigerated room at 0.5° - 3.5° C.

C. The horns were brushed onto a 9 cm filter paper disc and stored dry in the laboratory where the daily max. and min. were 25° - 27° C and 15° - 17° C respectively.

D. The horns were brushed onto an attached living juvenile leaf of *E. bicostata*. This leaf was infected with *P. eucalypti* but all or at least most of spores on it were removed by dipping it in water and brushing lightly. This leaf was allowed to dry and the spore horns from the filter paper sectors brushed onto the upper surface. The plant was kept in the laboratory as for the treatment C.

Under these conditions few if any new spore horns were
produced on the infected leaf.

The germination capacity of spores stored under the various conditions was tested over a 168 day period. The germination experiments were set up in glass dishes using as the spore source aliquots of spore solution or brushing spores from a piece of filter paper or leaf tissue. These methods of obtaining the spores for germination meant that the concentration of spores in the germination media varied. In all cases, by a haemocytometer count, the concentration of spores exceeded 32,000 per millilitre. The dishes were incubated and sampled as described previously. The germination was assessed by the ontogenetic scoring method.

The results are plotted in Figure 2/4 and presented in tabular form in Appendix 2.

The spores stored in the cold room in solution showed a significant reduction in germination only between 112 and 168 days after collection. By contrast the spores stored in any of the other treatments showed significant reductions in germination at 7 days after collection. At 7 days after collection the spores stored dry in the cold room had a significantly higher germination than those stored dry or on infected leaves in the laboratory. Spores stored on infected leaves do not retain their viability better than those stored on dry filter paper in the laboratory.
Mean ontogenetic germination score for 25 spores

Time (days)
Spore longevity has been reviewed in considerable detail (Cochrane, 1958; Hawker, 1950). As would be expected, different spore types have different survival capacities. In general, asexual spores are relatively short-lived, although some survive for long periods. (Cochrane, 1958) 

Germination of spores of _P. eucalypti_ as affected by age and method of storage (McMillan and Hunkin, 1951). Spores stored in solution in refrigerated room at 0.5°-3.5°C is likely that such spores will be more sensitive to temperature fluctuations. Spores stored dry in refrigerated room at 0.5°-3.5°C during incubation produce thick-walled chlamydosporic spores, which suggests that spores stored dry in laboratory on filter paper factors of the environment affecting spore survival are important. 

D. Spores stored on infected living leaf of _E. bicostata_ in the present experiment at low temperatures (Cochrane, 1958). It has been suggested that higher temperatures accelerate spore metabolism and exhaust reserves (Cochrane, 1958). In the present experiment storage dry at low temperatures gives a significantly lower longevity after 7 days by comparison with spore solutions at the same temperature. This illustrates the importance of relative humidity, which is often a critical factor in spore longevity (Cochrane, 1958). In leaves of susceptible hosts, it has been noted elsewhere that while the spores of _E. bicostata_ are highly susceptible to attack by _P. eucalypti_, the spores of _P. eucalypti_ are still viable.
Spore longevity has been reviewed in considerable detail (Cochrane, 1958; Hawker, 1950). As would be expected, different spore types have different survival capacities. In general, asexual spores of the Fungi Imperfecti are relatively short-lived, although some survive for long periods (Cochrane, 1958; Robert, 1948).

Septoria spores have a very thin wall (McMillan and Plunkett, 1942), and the wall of the Phaeoseptoria spore appears equally thin. It is likely that such spores will have a relatively low longevity under normal conditions. These fungi frequently produce thick-walled chlamydospores, these are possibly the means of long-term survival.

The factors of the environment affecting spore survival have also been reviewed, and as is the case in the present experiment, survival is longest at low temperatures (Cochrane, 1958). It has been suggested that higher temperatures accelerate spore metabolism and exhaust reserves (Cochrane, 1958). In the present experiment, storage dry at low temperatures gives a significantly lower longevity after 7 days by comparison with spore solutions at the same temperature. This illustrates the importance of relative humidity, which is often a critical factor in spore longevity (Cochrane, 1958).

It has been noted elsewhere that while the spores of
certain fungi survive longest at 75% relative humidity whatever the temperature (Naqui and Good, 1957) the sporidia of rusts, conidia of powdery mildew fungi and the sporangia of Phytophthora infestans survive longest at saturation (Cochrane, 1960). It had been hoped to evaluate the relative importance of temperature and humidity by storing spores in solution at room temperature. Under normal environmental conditions such spores would germinate and it was decided to store them at high pH levels which would suppress germination. The spores were kept in a medium of pH 8.0, resuspended in a pH medium of 4.0 by centrifuging, washing and addition of distilled water before setting up germination experiments. This experiment was unsuccessful, since the resuspended spores did not germinate even after only 48 hours exposure to the pH 8.0 medium. This was checked by storing stores in the cold room at pH 4.0 and pH 8.0 and comparing the germination of resuspended spores from each medium after 7 days. It was found that storage at the high pH level, even at low temperatures, apparently destroyed the spore viability or alternatively induced a type of dormancy which was not readily broken by heating, alternating hot and cold treatments or by exudates from the leaves of susceptible hosts.
Experiment 6  The effect of spore concentration on germination

Failure of crowded spores to germinate is a common observation (Boyd, 1952). At the other extreme from self-inhibition are examples like the ascospores of Ophiobolus graminis, which germinate slowly if isolated and more rapidly in heavy concentrations (Padwick, 1939). Some aspects of this phenomenon are probably related to the differences observed between washed and unwashed spores, and given in tabular form.

The following experiment was conducted to examine the effect of spore concentration on germination.

A very heavy spore solution was prepared by washing spores from infected leaves into distilled water. The spores were then washed and resuspended in distilled water. This solution was shaken on a mechanical shaker for 2 hours. An aliquot of the solution was taken after shaking the solution with an eye dropper and a drop placed on a haemocytometer slide. A count was made to assess spore concentration. 10 similar aliquots were taken and their concentrations assessed. The mean of these 10 observations was taken as the concentration in spores per ml. 5 ml of the original spore solution were taken, and after shaking for a further 15 mins on a mechanical shaker, made up to 10 ml with distilled water. The concentration of spores in this was again assessed.
with a haemocytometer. This procedure of sampling, dilution and assessment was repeated a further 6 times. A germination experiment was then set up in 8 glass staining dishes. Each dish contained .4 ml distilled water + .1 ml Potassium hydrogen phthalate buffer pH 4.0 + .1 ml spore suspension from one of the particular spore concentration suspensions prepared. The dishes were incubated, sampled as described previously and assessed by the ontogenetic scoring method.

The results are plotted in Figure 2/5 and given in tabular form in Appendix 2.

These results show that spore concentration in the germination medium affects spore germination even when spores are washed. This phenomenon is hence distinct from that discussed by other workers in relation to self inhibition (using this term in the narrow sense of materials present on, or produced by, spores which inhibit the germination of themselves or adjoining spores). The results show that a maximum germination is obtained when the spore concentration in the germinating medium is in range 258,000 to 16,000 spores per ml. Apart from the score at 16,000 spores per ml the readings show a fairly regular rise in germination with increasing spore concentration from 2000 to 32,000 per ml and a small fall once the concentration reaches 258,000 per ml. If it were critical, which was not the case in this investigation,
Mean ontogenetic germination score for 25 spores

Spore concentration (spores per ml. x 10³)
Germination of the spores of *P. eucalypti* in relation to the concentration of spores in the germinating medium.

Figure 2/5

A repetition of the experiment would be necessary to check the apparently anomalous value at 16,000 spores per ml.

Without further experiment discussion on probable causes of these results must be rather speculative. It is worth noting that the results could be due to the operation of single or multiple factors, resulting from spore concentration, affecting germination. If a single factor is postulated, then the curve obtained could be explained on the analogy of growth substance effect in the presence of excess of the substance. It would be fundamental to such a hypothesis to establish a) the level of spore concentration at which the germination score flattened out and b) that with concentrations above 258,000 increased inhibition of germination occurred. From previous work (Cochrane, 1956) on the effect of CO₂ concentration it seems possible this factor could be involved. By way of contrast if a multiple factor effect is postulated, the increasing germination with increased spore concentration could be due to a substance or substances and the drop at high spore concentrations due to lack of for example oxygen. (Spores of some fungi e.g. *Synchytrium endobioticum* are severely inhibited at 3% oxygen (Vladimirskaya, 1954).
In the present investigation the purpose of the experiment was to arrive at a conclusion which would lead to the necessary experiment and also if possible a range of concentrations over which spore concentrations could vary irrespective of experimen
t results without significantly affecting germination. Without further discussion on probable causes of these results must be rather speculative. It is worth noting that the results could be due to the operation of single or multiple factors, resulting from spore concentration, affecting germination. If a single factor is postulated, then the curve obtained could be explained on the analogy of growth substance effect on growth, i.e., an increase in growth with increasing concentration of the substance up to a certain level or levels and then an inhibition of growth in the presence of excess of the substance. It would be fundamental to such a hypothesis to establish a) the level of spore concentration at which the germination score flattened out and b) that with concentrations above 258,000 increased inhibition of germination occurred. From previous work (Cochrane, 1958) on the effect of CO₂ concentration it seems possible this factor could be involved. By way of contrast if a multiple factor effect is postulated, the increasing germination with increased spore concentration could be due to a substance or substances and the drop at high spore concentrations due to lack of for example oxygen. (Spores of some fungi e.g. Synchytrium endobioticum are severely inhibited at 3% oxygen (Vladimirskaya, 1954)).
In the present investigation the purpose of the experiment was to arrive at a spore concentration which gave maximum germination and also if possible a range of concentrations over which spore concentrations could vary in future experiments without significantly affecting germination.

The possible mechanisms of inhibition and promotion were not further investigated. In all future experiments the concentration of spores in the spore solution used was set at 400,000 - 600,000 per ml. Such solutions when diluted by germination medium, buffer etc., produced a range of spore concentrations, shown in this experiment to give the maximum germination. These levels were also within that range of spore numbers per ml over which no significant differences in germination occurred with changes in concentration.

2.9 Effect of pH on Germination

With air dispersed spores of fungi which parasitise the above ground parts of plants the effect of pH on spore germination is rather of academic than practical interest. It is unlikely that in vivo the pH of the host will be outside those limiting spore germination. Ecologically pH effects on parasitism by a fungus are more likely to be decisively expressed by affecting mycelial growth and spore formation than spore germination. This is true even if it is accepted that spore germination generally has a narrower
pH optimum range, than mycelial growth (Cochrane, 1958). This ensures in vitro studies on spore germination it is usual to conduct an experiment on pH because it is a possible variable which should be controlled in other treatments introduced into such studies.

A major complication in in vitro pH studies is the presence of buffering materials. A buffer is essential in such studies (Cochrane, 1958) otherwise the pH of the medium changes during the germination period. Some pH levels depend on the presence of buffering materials.

Two experiments were conducted to examine pH effects on germination. Experiment 1: The effect of pH on the germination of P. eucalypti, using various buffering materials.

In the first of these experiments, 4 ml distilled water + 1 ml washed spore solution of standardised concentration + 0.1 ml of appropriate buffer, were placed in each staining dish. The pH of each germinating dish peak at pH 4.5 which is significantly higher that at pH 2.9.

The buffers used were prepared from standard formula (Britton). After preparation, the pH levels were checked with a Jones' pH metre and cross checked with Universal indicator, to ensure consistency to within 0.2 pH units. The pH level of each buffer listed represents the average of 3 successive determinations on separate aliquots with the Jones' pH meter. At the conclusion of the experiment, the pH of each germinating
medium was checked with narrow range Merck pH papers. This ensured that the pH at the conclusion was within at least .5 pH units of its stated initial value. The buffers used and their pH value are given in the results. The treatments were sampled and assessed by the ontogenetic scoring method.

The results of this experiment are plotted in Figure 2/6 and presented in table form in Appendix 2.

The results bring out several points worthy of mention

1. The germination score at some pH levels depends on the buffer used to fix the pH e.g. compare the germination at pH 4.5, 2.5, 3.0, 5.0 and 5.5 in Citric Acid/Phosphate and Potassium hydrogen phthalate/NaOH/HCl buffer.

2. The shape of the curve is also affected by the type of buffer used. The Potassium hydrogen phthalate curve is a fairly classical type pH curve with a broad optimum lying between pH 3.5 and 6. The curve has a definite peak at pH 4.5 which is significantly higher than at pH 4.0 or 5.0. By contrast in the Citric Acid Phosphate buffer there are two optima, one between pH 2.5 and 4.5 and the other between pH 4.5 and 7.0. The trough at pH 4.5 is significantly lower than the peaks on either side. This result is particularly unexpected since pH 4.5 is the postulated peak in Potassium hydrogen phthalate NaOH buffer. In addition to a change in the peak of optimum
Germination of Spores of *P. eucalypti* at different pH levels using various buffering materials.

**Legend:**

- `x-x-x-x-x-x-x-x-` HCl/KCl
- `- - - - - - - -` K/H phthalate NaOH/HCl
- `- - - - - - - -` Citric Acid/phosphate
Mean ontogenetic germination score for 25 spores

pH
pH for germination with the two buffer systems, the limits of pH at which germination occurs are also different in the two buffers. A score of over 50 means that some germ tubes have been produced. At pH 2.5 in Citric Acid Phosphate the score is $52.6 \pm 1.21$ at the same pH in Potassium hydrogen phthalate buffer the score is only $42.0 \pm 1.22$. In other words on germ tube production, a commonly used method of defining germination, the lower limit of "germination" has probably already been passed at pH 2.5 in the Potassium hydrogen phthalate buffer but there is still "germination" at this pH in Citric Acid phosphate. From the shape of the two curves it is probable that the spores germinate at a higher pH in Citric Acid Phosphate than in Potassium hydrogen phthalate.

It has been frequently observed that different buffer systems affect the shape and limits of germination curves (Sussmann, 1954). The data above merely confirms that germination response to pH is dependent on the constituent materials used in the buffer. The occurrence of double pH optima for germination have also been recorded (Forbes, 1939). An exhaustive study of the growth of Coprinus sp. in relation to pH showed similar double optima curves. In that case the minimum between the two optima was shown to reflect pH dependent unavailability
of one or more inorganic elements. Although that study deals with cultural growth not germination, the explanation could well be similar with the above.

2.9 2 Experiment 8 The effect of buffer concentration on Spore germination of P. eucalypti

In this experiment the germination set up was as in the previous case except that the germinating medium consisted in each case of .4 ml of the appropriate buffer + .1 ml of distilled water + .1 ml of spore suspension. This experiment was carried out at the same time, under the same environmental conditions, using the same spore suspension and for the same germination period as Experiment 7. Hence a comparison between the data from the two sets of experiments can be made validly. The actual pH levels used are detailed in the results. The sampling and assessment of the germination treatments were as detailed previously.

The results are presented in Figs 2/7 and 2/8 and in Appendix 2 in tabular form.

The results show that germination is affected by the concentration of buffer used in the particular medium.

It will be noticed that in the media using Potassium hydrogen phthalate buffer the optimum germination is achieved at approximately the same pH level irrespective of buffer concentration. However where the concentration of buffer in
Germination of the spores of *P. eucalypti* at various pH levels in citric acid phosphate buffer at 2 concentrations.
Mean ontogenetic germination score for 25 spores

pH

Low Concentration
High Concentration
FIGURE 2/8

Germination of the spores of *P. eucalypti* at various pH levels in Potassium Hydrogen phthalate HCl/NaOH buffer at two concentrations.
Mean ontogenetic germination score for 25 spores

Low Conc.

High Conc.

pH
the germination medium is high there is a very sharp fall on either side of the optimum. The curve for the low buffer concentration tends to be flat topped. A perusal of these data shows that the second optimum at pH 4.7 is a single point. This is in accord with the results in this buffer at low concentrations, particularly since at both buffer concentrations the germination low point occurs at about the same pH level. The absence of a similar germination low point at the same pH level in the Potassium hydrogen phthalate buffer points to the materials used in the Citric Acid Phosphate buffer as being critical in showing up this pH effect.

These experiments confirm previous workers comments that the materials used in the pH buffer are of considerable significance in the effect of pH level on germination spores. In addition they show that the germination score is also dependent on the concentration of buffer used in the germination solution.

4 ml distilled water + .1 ml pH 4.0 Potassium

In all further in vitro experiments on germination, Potassium hydrogen phthalate buffer pH 4.0 at a concentration of .1 ml buffer to .4 ml treatment solution + .1 ml washed spore suspension was used.

2. 10. Effect of Temperature
Studies of spore germination, particularly of plant pathogens, in relation to temperature have been numerous (Hawker, L.E., 1950; Topa, 1949). A perusal of these data shows considerable discrepancies between the results of different workers for the same organism. The necessity for the control of other factors affecting germination before preparation of germination temperature curves has been emphasised (Cochrane, 1958). If factors other than temperature are limiting, the shape of the germination temperature curve will be considerably altered. The influence of suboptimal temperatures on germination appears to be essentially on the early processes of germination i.e., a brief exposure to the optimal temperature followed by exposure to suboptimal temperature will frequently give a final germination score, not greatly different from exposure to the optimal temperature for the whole germination period (Cochrane, 1958).

2. 10 Experiment Effect of Temperature on Germination

In this experiment 11 staining dishes were prepared containing 4 ml distilled water + 1 ml pH 4.0 Potassium hydrogen phthalate buffer. These dishes, set in separate petri dishes, were placed in the seed germination* cabinets. These cabinets are connected individually to a heating and refrigerating system. Although the cabinets are in a room having temperature control it is theoretically possible to maintain stable sub-ambient temperatures. The dishes were allowed to adjust themselves to the cabinet temperature and 1 ml of washed spore suspension was then added to each.

The results are plotted in Fig. 3/9 and presented in tabular form in Appendix 2. The temperature of the cabinets were read at 9 a.m. and 9 p.m. daily. The temperatures used in the results were the average of the temperature readings. The temperature control on these cabinets is not by any means perfect, and variations of ± 1 °C from the temperatures given were common in the early stages. The temperatures were recorded. The curve produced above a true optimum lying between 17.5 and 22.7 °C and slightly curved towards the right. This type of curve has been commonly observed (Chowdhury, 1966) in 1964. The point of maximum germination at 22.7 °C is not significantly higher than at 22.5 °C, which is the upper level extending from 20 °C to 22.5 °C, at a slightly suboptimal level. 

2. 10 Experiment 10 Effect of Temperature on Germination

These cabinets were located in the Division of Plant Industry C.S.I.R.O.
refrigeration system. Although the cabinets are in a room lacking temperature control, it is theoretically possible to maintain stable sub-ambient temperatures. The dishes were allowed 2 hours to adjust themselves to the cabinet temperature and 1 ml of washed spore suspension was then added to each dish. The dishes were incubated in the particular cabinets for 48 hours and sampled as described previously. They were scored by the ontogenetic scoring method.

The results are plotted in Fig. 2/9 and presented in tabular form in Appendix 2.

The temperatures of the cabinets were read at 9 a.m. and 4 p.m. daily. The temperatures used in the results are the mean of the 4 temperature readings. The temperature control on these cabinets is not by any means perfect, and variations of ± 1.5°C from the temperatures given, were common at the time when the temperatures were recorded. The curve produced shows a true optimum lying between 17.5 and 22.7°C and is slightly skewed towards the right. This type of curve has been commonly observed (Chowdhury, 1946; Yu, 1954). The apparent drop in germination at 27.2°C is not significantly lower than at 25.7°C. There is thus table top extending from 22°C to 27°C at a slightly suboptimal level.

2.10 Experiment 10 Effect of Temperature on germination

In view of the temperature variations in the cabinets
Germination of Spores of *P. eucalypti*

in relation to Temperature.
used in Expt 9, it was decided to carry out a further temperature germination test, this time using cabinets set in a cold room. The cold room had a temperature range of 0° - 3.5° C and the incubators set there in, showed no significant changes on a thermograph over a 24 hour period from their settings at 5°, 10°, 15° and 20° C. The above ambient temperature treatments of 25°, 30°, and 35° were carried out in incubators in the laboratory. The procedure of setting up the experiment was as described in Expt 9. The sampling of treatments was as described previously. In this instance, number of germ tubes per group of 25 spores was used as the measure of germination and 8 such groups of spores were taken for each temperature treatment.

The results are plotted in Fig. 2/10 and given in table form in Appendix 2.

In this experiment the optimum has been shown to lie between 10° C and 20° C and once again there is a suboptimal table top between 20° C and 30° C which shows uniform germination. These experiments taken together would indicate that the optimum temperature for germination lies between 10° and 22° C and is probably from 15° - 18° C. The minimum temperature for germ tube production is approximately 5° and the maximum above 35° C. The organism gives a suboptimal but good germination over a range of temperature from 20° - 30° C.
Germination of the spores of *P. eucalypti* in relation to temperature.

The results are plotted in Fig. 2/10 and given in Table I.

FIGURE 2/10
In further experiments on germination the spores were germinated at 25°C in an incubator in the laboratory except in the brief period of the year when the ambient room temperature rose above 25°C. At those times the germinations were carried out at 25°C in the cold room incubators. This temperature rather than the optimum was used because the incubator in the laboratory was of large capacity and could accommodate the large number of dishes used in some experiments. In addition 25°C lies on the table top area of the germination and variations of ±1°C from this temperature would have little effect on the germination achieved in different experiments.

2.11 Effect of Light on Germination

The significance of visible and ultra violet light in the germination of spores has been reviewed (Cochrane, 1960). Vitally stained spores can be rendered sensitive, presumably by photodynamic action, to visible light (Kaplan, 1950). In this instance sensitivity was estimated by mutagenesis. Inhibition of spore germination by visible light has been well documented in the rust fungi (Cochrane, 1945). With Physoderma maydis the shorter wave lengths of visible light were effective in promoting sporangia germination in the presence of oxygen (Herbert and Kelman, 1958). This is consistent with a possible role of photo oxidations. In the case of Peronospora tabacina visible light, in the intensity range
darkness to 460 f.c., did not affect germination (Shepherd, 1962).

An L.E. phytotron cabinet whose temperature could be regulated within ± 1.5°C, was available. This avoided a common difficulty in experiments of spore germination in relation to high light intensity; that of increasing temperature with increasing light intensity, (Gottlieb, 1950).

The spectral output of the T.L. - 33 tubes of the growth cabinets is highest in the 550-625 millimetrons wave band (Veen and Meijer, 1962), i.e. ultra-violet radiation is absent. Two germinating dishes were set up, each containing 4 ml distilled water and 1 ml 1/10 phthalate buffer pH 4.0, one placed in the phytotron cabinet at 25°C and the other in a dark incubator at 25°C for 2 hours to allow temperature adjustment. The dishes were seeded with washed spore solution and incubated for a further 48 hours under these conditions. Those in the cabinet were illuminated by fluorescent tubes supplying 3800 ft candles for 8 hours and complete darkness for 16 hours in each of the two x 24 hour days. The treatments were sampled as described previously and the number of germ tubes per group of 25 spores assessed.

The germination of fungal spores do not appear to have received any attention. By contrast conidiation in saprobes such as Absidia Caucasica, requires a light as well as a dark phase.
Germination of spores of Phaeoseptoria eucalypti in a dark incubator and in L.D. Growth cabinet at the same temperature.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean number of germ tubes per group of 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C in the dark</td>
<td>18.4 ± 1.89</td>
</tr>
<tr>
<td>25°C in growth cabinet</td>
<td>13.0 ± 1.15</td>
</tr>
</tbody>
</table>

Difference of 5.1% is significant at P 0.01

It has been long recognised that high intensity light inhibits spore germination (Hoffman, 1860). The significant depression of germination by the light treatment given in the phytotron cabinet accords with the results of effect of visible light on the germination of spores of the rust fungi (Cochrane, 1945).

From the viewpoint of fundamental effects of visible light on germination of Phaeoseptoria eucalypti spores the results do not allow any firm conclusion to be made. The light treatment given was of relatively high intensity and alternated with periods of darkness. These light alternations could have photoperiodic effects. Such possible effects in the germination of fungal spores do not appear to have received any attention. By contrast conidiation in Choanephora cucubitarum requires a light as well as a dark period (Barrett and Lilly, 1950).
period (Barret and Lilly, 1950). For the purpose of this study, the question of the effect of visible light on germination was not pursued. In all following in vitro germination experiments, the dishes were kept in the dark throughout the incubation period.

2. 12 General Discussion

From a perusal of literature, the ontogenetic germination scoring method, used in a number of the experiments in this thesis, does not appear to have been used before by pathologists studying germination. The essential reasons for using the method were 1) to overcome the observed and obviously potentially very large variability possible in germination counts on multicellular spores and 2) to allow to some degree for the potential infectivity of a spore which produces numerous germ tube tips. The method as used here, could result in a germination treatment in which no germ tubes were produced having as high or higher score than one in which the spores actually produced some germ tubes. This possibility does not occur in practice unless the spore populations used are very immature or non uniform. The series of points allocated to a spore are related to development towards germination. Hence a spore population which has some germ tubes will always have many of the other spores in its population on the way to germination, even if they do
not produce germ tubes in the time period of the experiment.

The method of scoring is essentially conservative in the
degree of difference indicated; this is important in assess-
ing the significance of a particular treatment in its effect
on germination.

The method could probably be improved by allocating
more than one point to a spore for the production of a germ
tube. This would emphasise the significance of the partic-
ular treatment in actual infection potential. It is also
possible that a differential system of points should be
allocated to spores with more than one germ tube. A spore
with one or two germ tubes, will usually have very long
germ tubes. A spore with 4 or 5 germ tubes, will frequently
have shorter tubes. The infection potential of the longer
as against the more numerous germ tubes would have to be
evaluated to arrive at a reasonable scoring procedure.

Since the foregoing experiments have all been discussed
individually, it will now suffice to indicate in discussion
& their significance in relation to one another and the
importance of the results in the ecology and pathology of
the organism.

The occurrence of an inhibitor in crude solutions of
spores, obtained by washing the spores from infected eucalypt
leaves has obvious adaptative advantage. The observation of
the satisfactory germination of spores in isolated spore horns indicates that the inhibitor probably comes from the leaf rather than from the spores. This mechanism would tend to prevent the spores from germinating on infected tissue. Since the infected areas on leaves are restricted by veins, the occurrence of the inhibitor in the localised areas of infection would satisfactorily restrict wastage of spores by germination on already infected tissue. It has been observed that if a drop of water is placed on a spore horn bearing infected area, the horn eventually collapses and as the drop dries, the spores form a mat on the infected surface. Under these conditions the spores do not germinate. If the mat is lifted from the infected area and placed in a drop of distilled water, germination occurs.

It has also been noted that when the spore horns are placed in distilled water they do not immediately break up, as is the case with *Septoria normae* (Heather, 1961b). The *Phaeoseptoria* horns desintegrate only if the solution is agitated. This could have adaptative significance. It is likely that this organism is mainly distributed by rain splash, as has been suggested for certain other pycnidial and perithecial forming fungi. (Ingold, 1953). If the horns tend to remain aggregated then splash droplets are likely to contain heavy spore concentrations and consequently
they will achieve the advantage of higher germination in heavy spore suspensions which is indicated in Expt 6. This suggestion requires further testing. The experiments reported do not allow the making of a clear cut distinction between the inhibition of germination by materials in crude spore solutions and the promotion of the germination of washed spores in solutions containing heavy spore concentrations. The hypothesis advanced is however consistent with the results of experiments conducted and observations on germination behaviour.

An alternative possibility is that the observed synergistic effect of spores in high concentration is purely a function of washed spores. The washing of spores in distilled water may result in leakage of materials essential to germination. When a great many spores are crowded together sufficient of the essential materials may be present to cause germination of some spores in the group. If such is the case the effect of enhanced germination with high concentrations of spores is not operative in the field situation.

The progress of germination with time indicates that some 28 + hours are necessary for a spore population to reach its maximum germination. This is a long while for a relatively thin walled spore to be exposed to the vagaries of the environment. An adaptation by the spore to any factor
speeding up the germination process would have obvious significance. This is further discussed in Chapters 3 and 4, and also be interpreted to only a limited degree. It has been shown that the survival of spores, even on infected host leaves, under laboratory conditions is relatively short. The conditions of this experiment unfortunately could not reproduce the normal field conditions. The juvenile leaves of the E. bicostata, are hypostomatous and the horns of P. eucalypti are exuded through the stomata. It is likely that the humidity conditions in such spore horns are very different from those achieved by placing the spores on the upper surface of the leaf as was the procedure in the experiment. The indications from the experiment are that humidity is a major factor in the longevity of the spore.

The experiments on the effect of pH on germination have little ecological or pathological significance. It has been shown that the pH of expressed leaf sap is not affected by the pH of the nutrient solution in which the plants are grown (Arnon and Johnson, 1942). It can be assumed that the pH of the leaf surface liquids on most plants will be within the range satisfactory for spore germination. Effects of pH on germination of P. eucalypti on leaves are likely to be via the effect of concentrations of materials which result in certain pH levels and the availability of certain essential
The likely effect of temperature on germination in vivo can also be interpreted to only a limited degree. It has been suggested that the effect of temperature in germination is largely due to its significance in the early stages of germination (Cochrane, 1958). Apart from the high mountain country, day temperatures in Australia, where eucalypts occur would normally reach for several hours the table top range shown in the experiment to give a high if not optimum germination. If temperature is a significant factor in the field distribution of *P. eucalypti* then it is unlikely that this is achieved through its effect on spore germination. In the field the organism most commonly occurs on the north coast of N.S.W. and Southern Queensland. It seems likely that in these areas satisfactory temperatures are combined with relatively high humidities and the latter may be the significant ecological factor. This likelihood is emphasised by the spontaneous occurrence of the organism on seedlings in nurseries and glasshouses of the drier western slopes of N.S.W. The sharp fall in germination which occurs above 30°C may account for the relative absence of the organism in glasshouses in these areas during the summer months. Experiments were conducted using the environmental conditions indicated. The *in vivo* significance of the effect of light on
Germination can also be assessed to only a limited degree from the experiment conducted. The light level in the phytotron cabinet, 3,800 ft. candles, is much lower than the level to which mature tree crowns are normally exposed. It is impossible to suggest, without experiments, what is likely to be the light intensity on the underside of eucalypt leaves or on pendulous leaves receiving most of their light by transmission or re-radiation. Undoubtedly the light levels on the forest floor of high quality eucalypt forest are much lower. Some measurements conducted in E. fastigata indicate light intensity on the floor of a fully stocked stand rarely exceeds 500 ft. candles (Cameron, 1964). It can be expected that under such conditions the germination of the spores of P. eucalypti will be much higher than in full sunlight and may even equal that in complete darkness. It could be expected that the disease would assume its greatest importance under these conditions and in crowded nursery beds where light levels on the under sides of leaves are also likely to be low. Under such conditions the effect of light intensity may be of considerable ecological importance.

As indicated in the individual discussions of experiments carried out in this chapter, future in vitro germination experiments were conducted using the environmental conditions indicated herein to be approximately optimum.
**CHAPTER III**

**Germination of Spores in vivo**

3.1 **Introduction**

In vivo studies of spore germination are much less frequent than in vitro ones, despite the fact that an in vivo study obviously has more possible immediate application in the case of plant pathogens. The comparative difficulty of carrying out such studies is a probable reason for their relative scarcity.

The early work on exosmosis by petals and leaves into drops of water suspended on them (Brown, 1922) has provided basic evidence of the significance of host organ exudates on the germination of plant pathogens. The importance of volatile materials produced by hosts has also been indicated (Brown, 1922a). The overall significance of biological stimulation of germination (whether of spores, seeds or pollen tubes) has been reviewed (Brown, 1946).

Reduced in vivo germination of the spores of *Uromyces phaseoli* on *Phaseolus vulgaris* leaves as influenced by the adjunct presence of rusted leaves has been considered as an explanation for the type of acquired immunity demonstrated in these locally lesioned leaves (Yarwood, 1954). More recently the significance of distilled water washings of tobacco leaves in the in vivo germination of *Peronospora tabacin* has been considered (Kry, 1962).

3.2 **Experiment 11 Effect of living host material on germination of spores**

A preliminary experiment was set up to test the significance of the presence of host material in spore germination. The three lowest fully expanded leaves of *Nicotiana* were detached from a seedling with a seedling knife. Three petri dishes were taken, the bottoms covered with vermiculite and a filter paper placed over this. The vermiculite and paper were soaked with distilled water. The petals of the leaves were poked through the paper and placed on the vermiculite with the underside of the leaf uppermost. Three microscope slides were placed in similar dishes as controls. The leaves and slides were then sprayed with distilled water until the vermiculite was running off. In all cases the slides remained until the vermicular was running off.

The covers were placed over each dish and the dishes incubated at 25°C for 48 hours. At the conclusion of this incubation period the slides were rubbed with a hot cloth to remove the vermiculite and the spores germinated.
explanation for the type of acquired immunity demonstrated in these locally lesioned leaves (Yarwood, 1954). More recently the significance of the distilled water washings of tobacco leaves in the in vivo germination of Peronospora tabacina has been demonstrated (Shepherd and Mandryk, 1962).

3.2 Experiment 11 Effect of living host material on germination of spores

A preliminary experiment was set up to test the significance, if any, of the presence of host material in spore germination. The three lowest fully expanded leaves of E. tereticornis were detached from a seedling with 12 leaf pairs. Three petri dishes were taken, the bottoms covered with vermiculite and a filter paper placed over this. The vermiculite and paper were soaked with distilled water. The petioles of the leaves were poked through the paper and into the wet vermiculite with the underside of the leaf uppermost. Three microscope slides were placed in similar dishes as controls. The leaves and slides were then sprayed with a fresh, washed, spore suspension adjusted to pH 4.0 with Potassium hydrogen phthalate buffer. In all cases the spraying was continued until the suspension was running off the slide or leaf. The covers were placed on the dishes and the dishes incubated at 25°C for 48 hours. At the conclusion of this incubation period the slides were dried with a hot
Results:

<table>
<thead>
<tr>
<th>Replicate No.</th>
<th>On Leaves of E. tereticornis</th>
<th>On glass slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.5 ± 2.6</td>
<td>1.7 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>23.0 ± 2.9</td>
<td>1.9 ± 0.05</td>
</tr>
<tr>
<td>3</td>
<td>23.2 ± 2.0</td>
<td>2.2 ± 0.13</td>
</tr>
<tr>
<td>Mean</td>
<td>23.2 ± 2.5</td>
<td>1.9 ± 0.04</td>
</tr>
</tbody>
</table>

TABLE No. 3/2 1 Germination of the spores of *P. eucalypti* on surface of leaves of *E. tereticornis* and on glass slides under the same environmental conditions.
fan, drops of Lacto-Phenol-Cotton Blue placed on them and covered with a large cover slip. The leaves when removed from the dishes were also surface dried with a hot fan cleared and stained by the process described in Chapter 1.

A piece approximately 6 cm x 1 cm, free of midrib, was cut from each leaf, placed on a microscope slide, a few drops of clear lacto-phenol placed on top and covered with a cover slip. Counts were made of the number of germ tubes per group, in eight x 25 spore groups on each slide and on each leaf piece. The counts were made moving over the slide or leaf piece in a regular pattern.

The results are given in Table 3/2 1.

The differences between the replicates of leaves and slides are not significant whereas the mean germination on the leaves is some 10 times that on the slides (significant at P .001). In addition the germinated spores on the leaves differed qualitatively from the germinated spores on the slides. The spores were longer, swollen and the germ tubes numerous and large on the leaf pieces (Plate 3/1).

The stimulation of germination of the spores by the presence of active independent tissue is in accord with the general thesis advanced by Brown (1946). On the other hand it is in contrast to the germination of Peronospora tabacina on Nicotiana tabacum C.V. Virginia Gold (Shepherd and Mandryk, 1962).
PLATE 3/1

Top: Germination of spores of *P. eucalypti* on leaf surface of *E. tereticornis*.

Lower: Germination of spores of *P. eucalypti* on glass slide (same period as above on leaf surface.)
These results pose some interesting questions e.g. -

**a.** Is the stimulation of germination characteristic of leaves of a number of eucalypts or is it a characteristic of particular eucalypt species or groups?

**b.** If the stimulation is widespread in the genus *Eucalyptus*, is it achieved by the leaves of other unrelated plants?

It has been observed that the stimulation of "germs" (sensu R. Brown, 1946) is not necessarily a property limited to hosts of the germ (be it spore or seed).

**c.** Is the stimulation or germination affected by spore age?

It has been observed that the stimulation of old spores is greater than of young spores in the case of some apparently volatile materials, (Brown, W., 1922).

Before proceeding to investigate some of these questions it was necessary to test—

1. If the stimulation of germination varied on different parts of the same leaf, different leaves of the same plant and on different plants where the host has been raised in a uniform environment.

2. It is possible that the effect is a contact stimulus on i.e. the spores make a better or a different kind of contact with leaves than they do with glass slides.

Thigmotropic effects in fungal penetration have received attention (Dickinson, 1960).
3.3 Experiment 12: The significance of between plants, within plants and within leaf differences in their effect on promotion of germination of spores.

To test the position effects mentioned, 3 plants of *E. grandis* were taken. These plants had been raised in Ceres phytotron in a uniform environment (phototemperature 27°C, nyctotemperature 22°C, 8 hours daylight, 8 hours fluorescent light, 8 hours night, watered with modified Hoagland's nutrient solution each morning and demineralised water at night). These plants had reached a height of 4'6" in 2½ months.

From each plant, the following leaves were selected:

1. The lowest undamaged leaf (8th node above cotyledons)
2. The leaf closest to 2' above soil level (18th node)
3. The leaf closest to 3' above soil level (22nd node)
4. The highest fully expanded leaf (usually about 4'2" above soil level (28th node).

Each of leaves, as in the previous experiment, was placed underside uppermost in a 5" petri dish on wet vermiculite and filter paper. Each was sprayed with a spore suspension from an atomiser until the solution ran off the leaves. The leaves were covered, incubated at 25°C for 48 hours, cleared and stained as before. Each leaf was cut into 3 approximately equal sections and each section mounted on a separate slide in clear Lacto-phenol with a coverslip. 8 groups of 25 spores
(4 groups either side of the midrib) were then scored for number of germ tubes, for each leaf portion.

The results of this germination count of some 7000 spores have not been presented in detail, since there were no significant differences either between different parts of the same leaf, different leaves on the same plant or between different plants. With this species at least, the germination score of the spore solution on part of a particular leaf will be characteristic of that spore solution for the leaves of the whole plant up to some 4'6" height.

In this experiment although the germination on the leaves was some 3-5 times the germination on slides which had been run as controls the germinated spores on leaves and slides were not qualitatively different. This indicates that all members of the Macranthereae (to which group both *E. grandis* and *E. tereticornis* belong) are not uniform in the stimulatory effect of their leaves on germination. *E. grandis* is a natural host for *P. eucalypti*, the original description of the pathogen having been made from leaves of this species, and these observations were a little unexpected.

3.4 Experiment 13 Germination of spores on the leaves of *E. bicostata* and *E. fastigata*.

It was decided to repeat the above experiment with modifications to eliminate to some extent the possibility
Germination of spores of *P. eucalypti* on the surfaces of the leaves of *E. bicostata*, *E. fastigata* and glass slides. Germinations on the leaf surfaces were computed for different parts of the same leaf.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>E. bicostata</th>
<th>E. fastigata</th>
<th>Glass Slides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Position on Leaf</td>
<td>Position on Leaf</td>
<td>Slides</td>
</tr>
<tr>
<td></td>
<td>Base of Leaf</td>
<td>Middle of Leaf</td>
<td>Tip of Leaf</td>
</tr>
<tr>
<td>1</td>
<td>24.5 ± 1.71</td>
<td>25.5 ± 1.52</td>
<td>1.34</td>
</tr>
<tr>
<td>2</td>
<td>23.3 ± .94</td>
<td>23.5 ± 1.63</td>
<td>1.45</td>
</tr>
</tbody>
</table>

* Each reading represents the mean score of 8 groups of 25 spores.
that contact stimulus might be a significant factor in the observed germination promotion. In this experiment 2 juvenile glaucous leaves of *E. bicostata*, 2 smooth juvenile leaves of *E. fastigata* were used, together with 4 glass microscope slides as controls. Drops of spore suspension had a very high contact angle with the waxy leaves of *E. bicostata* and the area of actual contact of the drop was reduced to a minimum. It had been observed earlier that juvenile and adult leaves of *E. bicostata* were natural hosts for the pathogen. The smooth green juvenile leaves of *E. fastigata* could be artificially infected with the pathogen. The contact angle of spore suspension drops on the leaf of this species was much lower than that on *E. bicostata* and a greater area of contact of leaf and drop was obtained. The leaves selected were from plants grown in the Ceres phytotron under identical conditions (those described in Expt 12) and the particular leaves were of the same ontogenetic age. (3rd leaf pair above the cotyledons). The leaves and slides were prepared and treated as before. After incubation for 48 hours at 25°C the leaves were cleared and stained. They were cut into sections and the germination scored as in Expt 12. These results are presented in Table 3/4 1.
In these results the differences between different parts of the same leaf and between different leaves from the same plant are not significant. The germination on leaves is some 5-6 times that on slides in the case of *E. fastigata* and some 10-11 times that on slides in the case of *E. bicostata*. The germination on *E. bicostata* is almost twice that on *E. fastigata*. The differences between the slides and leaves could still be a contact phenomenon, although, because of the magnitude of the difference, this is unlikely. The differences between the two species is almost certainly not the result of contact phenomenon since the species with the poorest contact (*E. bicostata*) has the greatest stimulatory effect on germination. There were qualitative differences between the germinations on the different media. The spores on *E. bicostata* were long and swollen with large germ tubes, the spores on *E. fastigata* and the glass slides were short, very little swollen with small thin germ tubes (Plate 3/2).

From these results and in view of the literature on exosmosis (Brown, *et al.*, 1922) it is reasonable to conclude that the greater stimulation on *E. bicostata* leaves compared with *E. fastigata* is due to a material, or concentration of a material, present in the drops on the former but absent from those on the latter. It is interesting to note that the leaves of *E. globulus*, a species closely related to *E. bicostata*,
Top: Germination of spores of _P. eucalypti_ on _E. bicostata_ leaf surface.

Lower: Germination of spores of _P. eucalypti_ on _E. fastigata_ leaf surface (some period as above).
showed promotion of germination of the spores of *Botrytis cinerea* apparently due to volatile materials present in the leaf (Brown, 1922). Such a result conforms with the general concept of biological stimulation of germination by living plant materials (Brown, 1946). In addition it appears to indicate a significant difference in stimulation by natural host and non-host species.

In the above experiment a comparison was being made between germination of the spores on leaves of the same ontogenetic age from two different eucalypt species. The above experiment was repeated using a greater variety of ontogenetic ages of leaf and including more eucalypt species.

**Experiment 14** Germination of the spores on the leaves of a number of *Eucalyptus* species. The leaves were taken from different positions on the plants of each species.

The following leaves were selected for use in this germination experiment:

- **E. bicostata** - 2 leaves from the 3rd node above the cotyledons
  
  " " - 2 " " " 5th " " " "

- **E. fastigata** - 2 " " " 3rd " " " "
  
  " " - 2 " " " 5th " " " "

- **E. robertsonii** - 2 " " " 3rd " " " "
  
  " " - 2 " " " 5th " " " "
TABLE 3/5 1 Germination of the spores of *P. eucalypti* on the leaves of three Eucalyptus species, one Eucalyptus hybrid and on glass slides (The leaves were selected from two nodal positions on each plant with one replication of each position)

<table>
<thead>
<tr>
<th>Nodal Position above cotyledons</th>
<th>Mean number of germ tubes per group of 25 spores*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3rd Node</td>
</tr>
<tr>
<td>Replicate</td>
<td>1st Leaf</td>
</tr>
<tr>
<td>Germination Media</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1st Leaf</td>
</tr>
<tr>
<td>E. bicostata</td>
<td>$26.8 \pm 1.67$</td>
</tr>
<tr>
<td>E. fastigata</td>
<td>$10.1 \pm 1.13$</td>
</tr>
<tr>
<td>E. robertsoni</td>
<td>$6.9 \pm 1.32$</td>
</tr>
<tr>
<td>E. fastigata</td>
<td>$8.8 \pm 1.61$</td>
</tr>
<tr>
<td>E. fastigata * E. robertsoni hybrid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1st replicate</td>
</tr>
<tr>
<td>Glass slides</td>
<td>$2.2 \pm .04$</td>
</tr>
</tbody>
</table>

* Each record on leaves represents the mean of 8 groups of 25 spores.
E. fastigata/- 2 leaves from the 3rd node above the cotyledons robertsoni hybrid

Glass slides -16 individual glass slides - 2 replicates of 8 slides each.

The same procedures of spraying, incubating, clearing mounting and assessing were used as in the previous experiments.

Results are presented in Table 3/51.

The same pattern is evident in this experiment as in the previous ones. The presence of independent active tissue promotes the germination of spores of P. eucalypti. The germination on E. bicostata leaves is over twice that achieved on either of the Renantherous species or the Renantherous hybrid used in the test. It is reasonable to conclude that this greater germination on E. bicostata leaves results from a promotory material and that this promotory material is either not present, or not at the same concentration, on the leaves of the other eucalypts used in this experiment.

However this material, or its concentration, are not the property of all species of the Macranthreae since E. grandis leaves in Expt 12 did not show the same quantitative superiority as a medium over glass slides. The germination on E. grandis leaves was not qualitatively different from those on slides or on Renantherous leaves. In all experiments with
E. bicostata and E. tereticornis leaves, the germination was qualitatively different from that on slides or on Renantherous species.

There is one other factor in these results which is worth noting. In the case of the leaves of all species the ontogenetic germination score is higher on the leaves from the lower than the upper node. This observation has significance in relation to susceptibility to infection of leaves at different levels.

3. 6 **Significance of the superficial coating on Eucalyptus leaves in the germination of spores of P. eucalypti.**

In some preliminary experiments on inoculation of E. bicostata seedlings with P. eucalypti it was noted that light wiping of juvenile leaves, which removed the surface wax (glaucous coating), was associated with a higher degree of infection. Investigations of other workers have indicated the presence of fungistatic and fungitoxic materials within angiosperm leaves (Gilliver, 1947) and also in the surface leaf exudates of certain woodland tree species (Topps and Wain, 1957). Work on the waxes on the surface of apple leaves has shown that some fractions of these are highly inhibitory to the germination of the conidia of Podosphaera leucotricha (mildew) (Martin Ball and Burchill, 1957).

It was decided to carry out an experiment to investigate
TABLE 3/6 1 1 The effect of removal of superficial wax from the surface of *E. bicostata* leaves, by light wiping with cotton wool, on the germination of the spores of *P. eucalypti*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean number of germ tubes per group of 25 spores</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwiped</td>
<td>25.56 ± 0.45</td>
<td>6.35</td>
</tr>
<tr>
<td>Wiped</td>
<td>27.26 ± 0.35</td>
<td>13.08</td>
</tr>
</tbody>
</table>

The difference of mean of 6.5% is significant at P < 0.001 (Student's T test).

TABLE 3/6 1 2 The significance of nodal position of the leaf on a plant of *E. bicostata* in the germination of spores of *P. eucalypti* on the leaf surface. (Unwiped leaves only were included in these results).

<table>
<thead>
<tr>
<th>Nodal Position</th>
<th>Mean number of germ tubes per group of 25 spores</th>
<th>Variance of a single observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>26.25 ± 0.51</td>
<td>6.35</td>
</tr>
<tr>
<td>4</td>
<td>26.71 ± 0.74</td>
<td>13.08</td>
</tr>
<tr>
<td>5</td>
<td>23.71 ± 0.92</td>
<td>20.48</td>
</tr>
</tbody>
</table>
Experiment 15 The effect of removal of the wax coating from *E. bicostata* leaves on the germination of spores of *P. eucalypti*

In this experiment a single 12" plant of *E. bicostata*, grown in the phytotron under the conditions outlined above, was selected. The opposite pairs of leaves at the 3rd, 4th and 5th node above the cotyledons were removed and one of each pair was wiped lightly with cotton wool and the other left unwiped. These leaves were placed in separate petri dishes inoculated, incubated, cleared and stained in the usual way. Each leaf was divided into 3 parts along the length and the midrib removed. The number of germ tubes per group of 25 spores for 8 groups was then assessed for each leaf portion.

These results are presented in Tables 3/6 1 1 and 3/6 1 2.

The mean number of germ tubes per 25 spores on each leaf section was calculated together with their standard errors. There was no significant difference between different sections of the same leaf so the 48 observations on each leaf were pooled. The data for the wiped leaves were then compared with those for the unwiped leaves pooling the data from all levels.
The data were then analysed for the significance of nodal position of unwiped leaves. The nodal position effect in wiped leaves was not considered at this stage, because it was possible that using the crude technique of wiping with cotton wool the removal of wax from different leaves might not be uniform.

The difference between node 3 and node 4 is not significant at P = .05 on student’s T test. The variance ratio Node 3/Node 5 is significant at P = .05 level, a Behrens modified T test was used instead of student’s T test for a comparison of means. The difference of means of 10.7% between Node 3 and Node 5 using this modified T test is significant at P < .05.

The variance ratio for Node 4/Node 5 is not significant at the 5% level, hence student’s T test was used to compare the means. The difference of 12.7% between the means Node 4 and Node 5 is significant at P < .05.

The removal of surface waxes from E. bicostata juvenile leaves over the range of the 3rd, 4th and 5th nodes results in an increase in the germination of the spores on these leaves. The results also establish the trend apparent in Experiment 14, lower leaves are more active in promoting the germination of P. eucalypti spores than upper leaves.

Experiments prior to this, indicated that there was a
material, either on, or which diffused from *E. bicostata* leaves, which promoted the germination of the spores of *P. eucalypti*. The significance of the above experiment in relation to this general conclusion could be explained by:

a. There is an inhibitor of spore germination present in the superficial waxy material of *E. bicostata*. The removal of this inhibitor results in greater germination on wiped than on unwiped leaves. This hypothesis could be extended to explain the nodal influences if it is assumed that as the leaf ages the effectiveness or quantity of the inhibitor is reduced, or

b. There is an inhibitor of spore germination present in the superficial waxy material whose removal results in higher germination on wiped than on unwiped leaves. In addition the quantity or effectiveness of the promoter increases with leaf age. This would explain the increased germination on leaves of the lower by comparison with the upper node.

If b. is correct then the same pattern of nodal effects should be evident in wiped leaves as well as unwiped. This is not the case. There is no significant difference between the germination on wiped leaves at different nodal positions in the data available. These data could be unreliable because of uncertain uniformity of wiping. To cover this
situation it would have to be postulated that in wiping the leaves the excess promoter on the leaves of the lower nodes was also removed or that the wiping was so non-uniform as to eliminate the effect of the excess on these leaves. It was not possible to express a definite opinion on either of these postulates. Hypothesis a seemed the simpler and was worthy of further testing in the first instance. In any case the establishment of the presence of an inhibitor in the surface waxy material was basic to both hypothesis. An experiment was designed to test this possibility.

3. 7 Significance of the ether soluble fraction of the superficial wax of E. bicostata leaves in the germination of the spores of P. eucalypti.

70 gms of glaucous juvenile leaves of E. bicostata were collected from plants grown in the previously described environment in the phytotron. 100 c.c. of Analar Ether was divided into equal parts in three beakers. Each leaf was then individually dipped, successively, into each of the three beakers containing the ether. This dipping removed the surface glaucousness from the leaves. The dipping time was only momentary so that as little material as possible from inside the leaf could diffuse out. The three ether dippings were then combined and reduced to 25 c.c. on a rotary film evaporator. The 25 c.c. of ether was then shaken with distilled water pH 6.0 in a separating funnel and the
Three successive shakings with 50 c.c., 25 c.c. and 25 c.c. water were carried out. The water, from three separations, was combined and the volume reduced to 7 c.c. on a rotary film evaporator at 40°C.

3.7.1 Experiment 16 The effect of the distilled water soluble fraction of the ether solubles of the superficial wax of E. bicostata leaves on the germination of spores

The water soluble fraction of the glaucousness was now tested in staining dishes using the technique outlined in the in vitro germination experiments, (Chapter 2). The ontogenetic scoring method was used to assess the germination.

The results are graphed in Figure 3/7.1 and given in tabular form in Appendix 3.

This experiment establishes that, at the concentrations used in the experiment, there is a water soluble inhibitor of spore germination present in the ether soluble glaucous coating of E. bicostata leaves. It became necessary, with the time available for the study, to make a choice between the inhibitor and the promoter of spore germination, present on eucalypt leaves, as a subject for more intensive study. The isolation and if possible characterisation of either of these materials would involve the same general approach. The promoter of germination was chosen for this study.
Germination of Spores of *P. eucalypti* in media prepared from the water soluble fraction of the ether solubles of the waxy coating of *E. bicostata* juvenile leaves.
Mean ontogenetic germination score for 25 spores

Percentage water soluble fraction of ether solubles on the surface of E. bicostata leaves in germination medium
TABLE 3/8 1 Germination of spores on the wiped and unwiped leaves of six species of *Eucalyptus*, one species of *Populus* and on glass slides.

Mean number of Germ tubes per group of 25 spores

<table>
<thead>
<tr>
<th>Germination Media</th>
<th>Unwiped</th>
<th>Wiped</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. tereticornis</em></td>
<td>16.0 ± 1.3</td>
<td>45.0 ± 2.8</td>
</tr>
<tr>
<td><em>E. bicostata</em></td>
<td>17.2 ± 1.6</td>
<td>42.3 ± 1.7</td>
</tr>
<tr>
<td><em>E. fastigata</em></td>
<td>7.3 ± .7</td>
<td>7.6 ± .8</td>
</tr>
<tr>
<td><em>E. pilularis</em></td>
<td>8.1 ± .7</td>
<td>8.5 ± .9</td>
</tr>
<tr>
<td><em>E. maculata</em></td>
<td>16.7 ± 1.2</td>
<td>10.9 ± 1.7</td>
</tr>
<tr>
<td><em>E. melliodora</em></td>
<td>6.9 ± .8</td>
<td>7.1 ± .7</td>
</tr>
<tr>
<td><em>Populus tremuloides</em></td>
<td>3.6 ± .4</td>
<td>3.2 ± .7</td>
</tr>
<tr>
<td>Glass slides</td>
<td>1.7 ± .04</td>
<td></td>
</tr>
</tbody>
</table>
(see Chapter 4) because a. it might give positive information on the reason for the limited host range of \( P. \) *eucalypti* in the field and b. there have been recent intensive studies carried out on the nature of inhibitory materials occurring on leaf surfaces (Shepherd and Mandryk, 1963; Martin et al., 1957).

3.8 **Experiment 17** Germination of spores on the wiped and unwiped surfaces of the leaves of a variety of *Eucalyptus* species

To conclude this series of in vivo germination experiments a test was set up using a variety of leaves wiped and unwiped, as germination media. The preparation, inoculation, incubation were those previously described. The germination was assessed by the mean number of germ tubes method.

The results are presented in Table 3/81.

These results emphasise the conclusions of the previous experiments:

a. By comparison with glass slides the presence of active independant tissues promotes spore germination.

b. That *E. bicostata* and *E. tereticornis* leaves stimulate germination of *P. eucalypti* spores to a much greater degree than do the leaves of a number of other eucalypts or the leaves of *Populus tremuloides*. *E. maculata* leaves unwiped show the same degree of promotion of germination as do the two Maeranthorous species.
c. In the case of *E. bicostata* and *E. tereticornis* light wiping with cotton wool and the removal of heavy glaucousness in the case of the former and a slight glaucous layer in the case of the latter, stimulates the germination of these spores still further.

d. The light wiping of the surfaces of other eucalypt leaves and the leaves of *P. tremuloides* does not stimulate germination of spores on the wiped compared with the unwiped leaves.

3.9 General Discussion

The overall results are in agreement with the general hypothesis that active independant tissues are significant in the germination of germs (Brown, 1946).

It has been established that the leaves of all species tested, stimulate the germination of the spores of *P. eucalypti*. This general stimulation of germination is in agreement with the observation that in the case of non-obligate parasites exogenous substrates are frequently a source of nutrient and stimulate the germination of such spores (Gottlieb, 1950, 1964). However there are definite indications of specificity of the independant active tissue in the qualitative and in the degree of quantitative stimulation achieved.

*E. bicostata* and *E. tereticornis* leaves have been shown to stimulate the germination of *P. eucalypti* spores to approximately twice the level achieved on the other species tested.
with the exception of E. maculata. Further they cause more obvious swelling and elongation of the spores. These two eucalypt species belong to the subgenus Macranthera of the eucalypts (Pryor, 1959), and are both parasitised by the organism in the field. Members of the subgenus Renanthera stimulate the germination of the spores of the organism but at a lower level. These hosts can be artificially infected with the organism. There is a nursery record of the organism occurring on E. maculata, a member of the Corymbosa (Walker, 1962), and the leaves of this species show a high degree of germination promotion. In artificial inoculation experiments in the glasshouse the parasitism of members of the subgenera Renanthera and Corymbosa is much less severe and natural infection in the glasshouse is negligible.

High stimulation of germination, whether considered qualitatively or quantitatively, is not the property of all members of the Macranthera. Leaves of E. grandis, the host from which the organism was originally described, show a germination level which is qualitatively and quantitatively similar to the leaves of members of the other subgenera of the genus Eucalyptus. In the glasshouse E. grandis is readily infected with the pathogen by natural and artificial inoculation. It may be that an additional factor is important in the degree of susceptibility in this instance e.g., the leaves
of *E. grandis* are usually readily wet by a spray of spore solution while the leaves of *E. bicostata* and *E. tereticornis* are difficult to wet, unless the leaves are first wiped with cotton wool. (The correlation between spore deposition and degree of infection is considered in Chapter 5). Greater wettability of leaves results in greater spore loads deposited from the same amount of spore solution. In other words, the "inoculum potential" (Garrett, 1956) of the spore solution is higher in this case; this could be the critical factor.

Although the overall effect of the leaves of *E. bicostata* and *E. tereticornis* on germination is highly promotory, the degree of stimulation can be still further increased by light wiping of leaf surfaces of these species with cotton wool. The glaucous material removed by such wiping in the case of *E. bicostata* leaves has been shown to contain a water soluble inhibitor of germination, which was very active *in vitro* at the concentrations used in the particular experiment. The presence of materials inhibitory to germination on leaf surfaces has been demonstrated elsewhere (Martin et al., 1957; Topps and Wain, 1957; Shepherd and Mandryk, 1962).

The presence of such inhibitors on leaf surfaces has obvious ecological and pathological implications which have been discussed elsewhere (Martin et al., 1957; Wood, 1960).

(See Studies of the waxes of *Eucalypts* and certain other...
Australian plants have shown that long chain B-diketones may form up to 50% or more of the total wax on these leaves (Horn and Lamberton, 1962). Further investigation of the surface waxes of glaucous eucalypt species of the Globulares has shown the presence of B-Diketones. These can be almost quantitatively removed by light wiping with cotton wool (Lamberton, 1964). The physical arrangement of the waxes on eucalypts has also been discussed (Lamberton, 1964; Hall, Chambers and Hallam, 1964). Various possible functions have been suggested for the glaucous waxes of eucalypts and the consequent ecological significance discussed (Barber and Jackson, 1957). The inhibition of insect and fungal attack was among the suggested functions. The significance of the waxy coating on eucalypt leaves is further discussed in Chapter 5.

It has been demonstrated that the lower leaves on E. bicostata give a greater stimulation to germination than do the upper leaves. In this instance no attempt has been made to distinguish between ontogenetic age and physiological age of the particular leaves. It is worthy of note that this picture of germination stimulation is positively correlated with the degree of infection achieved on leaves of a plant whether the infection is natural or by artificial inoculation. (See Chapter 5).
As mentioned earlier, this difference in the degree of stimulation with nodal position could be due to the presence of less inhibitor on older leaves or to the presence of greater quantities of, or more active stimulatory materials on older leaves. No attempt has been made to discriminate between these possibilities. It is worthy of note, that in the case of Blue Mould of Tobacco, it has been found that the amount of material inhibitory to the spore germination of the causal pathogen (*Peronospora tabacina*), increases at successive nodes up the plant. It has also been shown that this increase is negatively correlated with the susceptibility of the leaves at successive nodes up the plant to infection. (Shepherd and Mandryk, 1963). It has also been shown elsewhere (see Chapter 5) that the weight of ether soluble materials per unit leaf area is significantly greater on the three topmost by comparison with the three lowest pairs of leaves on *E. bicostata* plants with twelve leaf pairs. Determination of the variation in the level of germination inhibitors on leaves at different positions on *E. bicostata* plants would be fundamental to understanding the pathology of *P. eucalypti* on these leaves.

3. 10. Conclusions

1. The germination of the spores of *P. eucalypti* on detached leaves of various *Eucalyptus* species and of *Populus*
tremuloides is stimulated by comparison with germination on glass slides.

2. The germination of spores of P. eucalypti is significantly higher quantitatively and qualitatively different on E. bicostata and E. tereticornis juvenile leaves than on the leaves of other eucalypt species examined, except for E. maculata which shows comparable quantitative germination promotion. Light wiping of E. bicostata and E. tereticornis leaves removing the waxy coating, further stimulates germination.

3. If the juvenile leaves of E. bicostata are lightly wiped, the germination of the spores of P. eucalypti is 6.5% higher on wiped leaves than on unwiped comparable leaves.

4. The germination of the spores of P. eucalypti on detached unwiped juvenile leaves of E. bicostata taken from the 3rd node above soil level is 10.7% higher than on leaves taken from the 5th node above soil level.

5. The germination of spores of P. eucalypti on detached juvenile leaves of E. bicostata taken from the 4th node above soil level is 12.7% higher than on leaves taken from the 5th node above soil level.

6. If the juvenile leaves of E. bicostata are dipped in ether to remove ether solubles from the surface, the water soluble fraction of the ether soluble materials is
inhibitory to spore germination at the concentrations used in the germination media in the particular experiment.

Investigation of the Promotion of the germination of spores of $F.\_eucalypti$ by the water soluble leaf exudate of $E.\_bicostata$ has not been demonstrated by studies in the literature to date. The ability to stimulate the germination of spores of $F.\_eucalypti$ was quantitatively higher and qualitatively different on $E.\_bicostata$ and $E.\_teretiformis$ juvenile leaves, from that on the leaves of most other species used in the experiments, as illustrated by the details presented in Chapter 3. It has been suggested that, as a corollary to the "recognition" of a suitable host substrate by external chemical stimuli, has no particular survival value, but rapid internal organization to form infection structures in response to contact with a solid surface would seemingly have considerable advantage (Blatje, 1959). This suggestion will be discussed more fully at the end of this chapter. It is probably sufficient to note here that its applicability
Investigation of the Promotion of the germination of the spores of *P. eucalypti* by the water soluble leaf exudate of *E. bicostata* has been demonstrated as a Plasmodesmopora brassicae (Macfarlane, 1952). The ability of airborne fungi, unlike soil borne fungi, reach their substrate passively by air movement. It has been suggested that, as a corollary to "recognition" of a suitable host substrate by external chemical stimuli has no particular survival value, but rapid internal organisation to form infection structures in response to contact with a solid surface would seemingly have considerable advantage (Flentje, 1959). This suggestion will be discussed more fully at the end of this chapter. It is probably sufficient to note here that its applicability
in relation to a particular airborne pathogen, will depend on the inoculum potential of the individual spore of the pathogen and the degree of specialisation which the organism shows in its parasitic relationships.

Promotion of germination of the spores of soil fungi by different chemicals, secreted by hosts and non-hosts, has been demonstrated for relatively specialised parasites such as *Plasmodesmata brassicae* (Macfarlane, 1952). The ability of non-host plants to stimulate the germination of spores of *Spongospora subterranea* has been used as a means of control of powdery scab of potato by decoy cropping with *Datura stramonium* (White, 1954). Similarly, the germination of the conidia of *Fusarium sp* (Jackson, 1957) and of the oospores of *Pythium* (Barton, 1957) has been shown to be stimulated by the close proximity of certain host roots. In the former case the germ tubes showed a strong tropic growth towards the roots. To some extent promotion and inhibition of the germination of spores of soil fungi under field conditions can be regarded as an extension of the rhizosphere effect (Lockhead, Timonin and West, 1940). In any particular germination study it is also necessary to appreciate the generalised conclusion on the significance of external substrates in the germination of spores of obligate and facultative parasitic fungi (Gottlieb, 1964).
The significance of specific exudates of leaves in the germination of airborne fungi has received less attention. The results of the early work on the exosmosis in leaves and petals in the germination of Botrytis sp. could have reflected a substrate effect (Brown, 1922). The observations on the fungitoxicity of certain ground up exudated of angiosperm leaves (Gilliver, 1947) and of surface exudates of leaves of certain woodland species (Topps and Wain, 1957) were not pursued to isolate the active chemicals involved. The chemical investigation of the inhibition of germination of Podosphaera leucotricha by the waxy materials on the surfaces of apple leaves has shown that the ether soluble acidic materials, which constitute some 40% of the wax, are the most active fraction in the inhibition (Martin et al, 1957).

More recently the investigation of the factor on the surface of tobacco leaves which inhibits the germination of Peronospora tabacina has resulted in the chromatographic isolation of some seven areas of biological activity all of which corresponded to the presence of phenolic compounds (Shepherd and Mandryk, 1963).

In the investigation which follows the aim was to isolate and purify as far as possible, the material responsible for the promotion of the germination of the spores of P. eucalypti by the leaves of E. bicostata. The leaves of
E. bicostata and E. tereticornis had both shown considerable promotion of the germination of the spores of P. eucalypti over and above that achieved on most other host and non-host leaves. It seemed reasonable to assume that this additional promotion was of a more specific type than the general promotion of germs by living substrates (Brown, 1946), and that it could be a significant factor in the ecology and pathology of the organism.

4.2 Results of Preliminary Investigations

An investigation of the promotion of the germination of spores by plant materials involves a long series of experiments, the design and constitution of each experiment depending on the results of those preceding it.

Before attempting to isolate a particular "promoter" it is necessary to carry out certain preliminary experiments. The design of these experiments has a primary purpose a more precise definition of the aim of the investigation e.g., age of leaves on which promotion occurs. These experiments also aim at eliminating a number of possible chimeras of promotion e.g., the promotion may be a function of pH of the medium, or be a pH/promotive material interaction, or the promotion may be a function of time. At this stage it is desirable to know something of the nature of the promotive material e.g., its heat stability, useful for its own sake but also as an
indication of general stability. In addition it is desirable to find out whether the material causing promotion is dialysable. This technique achieves a crude separation of the promoter from the plant extract. It also indicates the upper limit of molecular weight of the promoter. Finally if the promotion is apparently due to a dialysable material then an attempt should be made to eliminate the possibility that the material is an inorganic nutrient ion.

In this investigation ten experiments were performed to satisfy these basic requirements. These experiments are reported in detail as preliminary investigations in the Experimental addenda section. The general conclusions arrived at were:

1. Promotion of the germination of spores of P. eucalypti was quite as effective by mature as by juvenile leaves of E. bicostata.

2. Significant promotion occurred over a wide range of pH and in two pH buffers of different constitution.

3. The promotion was not a simple time effect. The time course of germination in the leaf extract showed an earlier commencement of germination and a higher total
germination than in distilled water at the same pH.

4. The promotion factor was heat stable - the extract from the leaf could be boiled without impairing its efficiency.

5. The promotion factor was dialysable.

6. The promotion factor was probably not due to an inorganic nutrient ion.

4. 3 The isolation and purification of the material from *E. bicostata* leaves responsible for the germination promotion of *P. eucalypti* spores.

4. 3 1 Introduction

Following these preliminary investigations a series of twenty six experiments were carried out to isolate and purify the promotive factor in the leaf extract. The details of method, results and discussion of each experiment are presented in the Experimental Addenda section under Intensive Investigation of the factor causing major promotion of spore germination.

4. 3 2 Materials and Methods

*In vitro* germination experiments used the same experimental materials as those used in similar experiments in Chapter 2.

The techniques of separation used involved

a. Dialysis

b. Solubility in the following series of organic solvents
1. Petroleum ether
2. Ethyl acetate
3. Ethyl acetate
4. n-Butanol saturated with distilled water-organic
5. 80% isopropanol distilled water
6. 40% acetone distilled water
7. Petroleum ether
8. Ether
9. Benzene
10. Chloroform
11. Acetone
12. Dcnzene
13. Sodium hydroxide
14. Chloroform
15. Paper chromatography on Whatman's No. 1 chromatographic paper to locate areas of germination promotion in the chromatographed solvent soluble fractions, similarly due to hydrolysis by strong acid, into Paper chromatography on J.M.M. (heavy) Whatman's eluates were chromatographic paper, either singly or in room at 1-3°C. chromatopiles to yield adequate quantities of eluate to test the effect on in vitro germination. These paper chromatographic techniques were used the ontogeny with three successive solvent systems to achieve of germ tuber progressive purification of the active eluate.

The solvent systems used were

1. n-Butanol saturated with distilled water-organic to pH 4.0 and phase with the series of organic solvents of increasing degree of promotion. 40% acetone distilled water
2. The final separation and purification was carried out on thin layer chromatograms using silica gel water solution glass. The two solvent systems used were
of the ext.

1. 75% Benzene 25% Acetone

2. 65% Benzene 35% Acetone

Analogous ethanol was used as the agent to elute the materials from the chromatograms both paper and thin layer. The reduction of the volume of dialysed material and the drying off of organic solvent, solvent soluble fractions and of eluates of chromatograms were all carried out on a rotary film evaporator at less than 40°C. After some initial failures, apparently due to hydrolysis by strong acid or base, solvent soluble fractions, washed extracts and eluates were stored at pH 4.0. Storage was in a cold room at 1°-3°C. (active front) values for germination promotion in the

**In vitro** germination experiments whether in dishes or on pieces of paper chromatograms were generally assessed by the ontogenetic scoring method and occasionally by the number of germ tubes method (see Chapter 2).

### 4.3.3 Experimental Results

The dialysed water soluble leaf extract was acidified to pH 4.0 and washed with the series of organic solvents of increasing polarity, listed above. A significant degree of promotion of quantitative spore germination was shown by the ether and ethyl acetate soluble fractions of this extract. By contrast the promotion of germ tube length appeared to be due to water soluble materials. The petroleum ether soluble fraction
of the extract showed a significant inhibition of spore germination.

Preliminary germination experiments, using pieces of Whatman No. 1 chromatograms of the ethyl acetate soluble fraction of the extract, developed in Butanol: Acetic acid: water (4: 1: 5) organic phase and Acetic acid: Water (15: 85) showed that it was possible to achieve a reproducible separation of the constituent promoters and inhibitors of germination present in this fraction. A further experiment using the same paper, with n-Butanol saturated with water as the solvent, showed there was a general agreement in the R.F. (Relative Front) values for germination promotion in the following chromatographed solutions:

a. The original leaf extract
b. Original leaf extract after dialysis
c. Ether soluble fraction of the dialysed leaf extract
d. Ethyl acetate soluble fraction of the dialysed leaf extract
e. Residual water soluble fraction of dialysed leaf extract after washing with organic solvents

Whatman No. 1 paper chromatograms of the ethyl acetate soluble fraction of the dialysed leaf extract developed in n-Butanol saturated with water showed two areas of significant germination promotion (R.F. values .03 and .45). It was
shown, by comparative paper chromatography, in three solvent systems, that the area R.F. .03, which was associated with a light blue fluorescence under an ultra violet lamp (wavelength ca. 300 m.) corresponded with chlorogenic acid.

In vitro germination experiments using the purified acid, from two commercial sources, showed that Chlorogenic acid, at a concentration of 7 x 10^{-3} Molar, showed a promotion of spore germination of some 20% (Sig. at P < .001).

Twenty % 3 M.M. paper chromatograms of the ethyl acetate soluble fraction of the dialysed leaf extract were developed using n-Butanol saturated with water as the solvent system. The area of maximum promotion in these chromatograms, which corresponded to the R.F. value .49 in those above, was calculated and the area eluted. The eluate was successfully tested for germination promotion. This eluate was then chromatographed in a chromatopile of 3 M.M. papers using 80% isopropanol as solvent. The area of maximum germination promotion in these papers was located at R.F. .88-.98. This area was eluted and a satisfactory germination in relation to eluate concentration curve, was produced. This eluate was then rechromatographed in a chromatopile of 3 M.M. papers using 40% acetone as the developing solvent. In this solvent system maximum germination promotion was located at R.F. .76. The eluate of this R.F.,
zone, at its optimum concentration for germination, promoted spore germination by 68% (Sig. at P < .001). An experiment was conducted using Chlorogenic acid and this eluate separately and in combination. The combination of both materials gave a 10% (Sig. at P < .01) promotion above that of eluate alone and a promotion of 50% (Sig. at P < .001) above that of chlorogenic acid alone.

The eluate of the 40% acetone chromatopile was washed with ether and it was shown that the ether soluble fraction contained the promotive factor of spore germination. This ether soluble fraction of the eluate was chromatographed on Silica gel thin layer chromatograms developed in 75% benzene 25% acetone. Five fluorescent zones were produced. These were eluted separately with Analar ethanol.

A difficulty faced in these final stages of the investigation was the increasing insolubility of the eluted materials in distilled water. Eventually it was necessary to carry out germination tests on the eluates dispersed by ethanol in distilled water. Ethanol in distilled water at the same concentration was used as the "control". Tests of the eluted fractions of the thin layer chromatograms showed that a light blue fluorescent spot R.F. .31 in the 75% Benzene 25% Acetone solvent system gave a germination promotion of 23% (Sig. at P < .001) above that of the control. Significant promotion,
by eluates of this R.F. value, was reproducible when different spore solutions and new ethanol distilled water "controls" were used in a germination experiment.

This light blue fluorescent spot was moved to R.F. \(0.41\) by developing the chromatograms in 65\% benzene 35\% acetone. The eluate of R.F. \(0.41\) showed a germination promotion of 80\% (Significant at \(P < .001\)) above the comparable ethanol distilled water control.

Eluates of the other R.F. areas of these thin layer chromatograms did not show significant promotion of spore germination. Ethanol in distilled water at particular concentrations showed considerable promotion of germination above that in distilled water alone.

4.40 General Discussion

The experiments comprising this series have been discussed individually in relation to the main line of the investigation i.e. the isolation and purification of promoters of the germination of the spores of \(P.\) eucalypti (See Experimental Addenda). Further discussion is now limited to an overall review of the results and their significance in relation to the ecology and pathology of the disease, and completed with a series of conclusions which can be drawn from the experiments.

The \textit{in vivo} germination experiments of Chapter 3
indicated a significantly higher and qualitatively different germination of the spores of *P. eucalypti* on the leaves of *E. bicostata* and *E. tereticornis*. The experiments presented here have isolated Chlorogenic acid (which has been shown chromatographically to occur probably on the leaves surface of *E. bicostata*) and another more significant promoter of spore germination of *P. eucalypti* to be present in the water extract of *E. bicostata* leaves. It has been suggested, as mentioned in the introduction to Chapter 4, that recognition of a suitable substrate, by external chemical stimuli, has no particular survival value for airborne spores (*Fentje*, 1959). The survival value of a chemical stimulus by the host species to the germination of the spores of a particular pathogen probably varies considerably with the pathogen concerned. In the case of obligate biotroph spores it has been shown that germination is not significantly affected by the presence of external substrates (*Gottlieb*, 1964). Even if the germination of such spores were affected by external substrates the survival value of adaptation to a particular chemical stimulus would probably be low. The host germination of the spores of rusts, powdery mildews etc. is usually rapid - a matter of 2-10 hours. The individual spores of obligate biotrophs in general have a high inoculum potential - a single spore frequently being sufficient to
establish an infection (White, 1957). Consequently an adaptation for faster or more complete germination by a particular spore genotype in a mixed spore solution would probably have a relatively low survival advantage.

If an obligate biotroph is also an obligate stomatal penetrant or varies in its potential to produce penetrating structures, adaptation to physical or chemical stimuli favouring the direction of growth or the formation of such structures could have survival advantage. (Dickinson, 1949, 1960; Flentje 1959).

The situation with non-obligate biotrophs and necrotrophs could be very different. The germination period for such spores can be quite long - in the case of P. eucalypti 28+ hours. The inoculum potential of a single spore may be low, i.e. a considerable number of such spores may be necessary to establish a single infection (White, 1957). An adaptation to react to chemical or physical stimuli increasing total germination or, possibly more important, reducing the period for commencement of germination, could have considerable significance. In a mixed spore population, falling on a host leaf, those spores which react positively to such a stimulus will colonise the leaf in a variable environment. If this positive reaction to the stimulus is genetically fixed then it will be those spores which have this adaptive capacity.
which will be reproduced in greatest numbers. This is to be expected unless environment, during the long germination period of non-reactive spores, remains favourable for field germination and infection.

In the case of *P. eucalypti* with its long germination period the adaptation to react, to a chemical stimulus from a host, by faster and more complete germination of a spore population (See Expt. No. 5a, Experimental Addenda) probably has considerable significance.

A further point which follows from the above discussion is the extent to which such an adaptation contributes to host specificity in the genus *Eucalyptus*. The significance of the adaptative advantage can be expected to vary considerably on different species. In Chapter 5 it is shown that the surface of the leaves of *E. bicostata*, in particular, and of *E. tereticornis*, are hydrophobic. This affects the number of spores of *P. eucalypti* which are successfully deposited. In other words the hydrophobic nature of the surface reduces the inoculum potential of the spore load sprayed on the leaf. An adaptation which ensures the rapid and high germination of spores successfully deposited could be very important. On certain other species with lesser hydrophobic surfaces e.g. *E. grandis*, such an adaptation may have much less significance. In the case of *E. grandis*
although there is no evidence of germination promotion of the level of that observed in *E. bicostata* and *E. tereticornis*, infection is frequently successful. *(E. grandis* is a field host of the pathogen).

Apart from nursery records, the only successful infections of eucalypts outside the *Macranthera* have been by artificial inoculation with heavy spore suspensions. It is likely that these inoculations gave such high inoculum potential levels that they were sufficient to overcome the normal disease escape mechanism of these species in the field. It would be interesting to carry out inoculation of these groups with various spore concentration levels to determine threshold values of inoculum for successful infection of various species. This should make the situation clearer and might high-light the importance of germination promotion on certain species for successful infection following inoculation.

Further investigation would be necessary to establish the host specificity of the germination promoters as a prerequisite to postulation of their significance in host specificity to infection. Chlorogenic acid is a material of widespread occurrence in plants and is consequently unlikely to give qualitative host specificity. The major promoter of spore germination has not been chemically characterised, consequently its host specificity cannot be assessed. The materials which are soluble in organic solvents.
experiments indicate that the concentration of the promoter in contact with the spore may be the important factor in host specificity.

The situation is further complicated because the extract of the leaves of *E. bicostata* consists of a mixture of inhibitors and promoters. It is possible that it is the balance of these, at particular concentrations, in contact with the spore which could provide the basis of host specificity. The elucidation of this balance would require a great deal more investigation of the type pursued in this series of experiments.

### 4.50 Conclusions

1. The water soluble extract of the mature leaves of *E. bicostata* whether these leaves are in whole sections or in cut up pieces, has an overall promotory effect on the germination of the spores of *P. eucalypti*.

2. This promotory effect results from materials some of which at least are dialysable and heat stable, even surviving boiling to dryness.

3. Little if any of the promotion in the leaf extract can be attributed to inorganic ions unless the spores happen to require the inorganic ions in a chelated form - it seems impossible at present to eliminate this possibility.

4. The promotion appears to be partly due to organic materials which are soluble in organic solvents.
5. A proportion of the germination promotion materials are soluble in both ether and ethyl acetate. The promotion factor of germ tube elongation by contrast appears only to be water soluble.

6. The ethyl acetate and ether soluble fractions of the dialysed *E. bicostata* leaf extract, when chromatographed show areas of germination promotion which correspond to areas of promotion in the chromatogram of the crude dialysed leaf extract.

7. From the chromatograms of the dialysed extract, the ethyl acetate and the ether soluble fractions of the extract, it is apparent that the leaf extract, although showing an overall promotion of germination, consists of a complex mixture of inhibitors and promoters and perhaps neutrals.

8. The Chromatogram of the ethyl acetate soluble fraction shows two distinct areas of promotion. One of these areas has been shown to correspond chromatographically to Chlorogenic acid. A germination test using pure Chlorogenic acid showed that this acid at a concentration of $7 \times 10^{-3}$ molar shows a promotion of the germination of the spores of *P. eucalypti* of approximately 25%.

9. An apparently more significant promoter of spore germination has also been isolated from the ethyl acetate
soluble fraction. A promoting material has been obtained chromatographically pure and this possibly consists of a single chemical entity. This material after being isolated and purified by a series of paper and thin layer chromatograms in various solvents, causes a promotion of up to 230% in the germination of the spores of *P. eucalypti*. A major difficulty in working with this material is that since its chemical constitution is unknown it is not possible to specify the molar concentration which gives this high degree of promotion. A further difficulty, with the purified material, was its insolubility in water. When chromatographically pure a bio assay of it in distilled water, using *P. eucalypti* as the test organism would indicate that it is insoluble. For purposes of assessment it had to be dispersed in distilled water. This dispersion was made in ethanol which was shown to be a promoter of spore germination at certain concentrations.

10. Although, as mentioned above, it is not possible to specify the molar concentration of the major promoter, which gives the optimum promotive effect, some idea of the order of this concentration can be calculated. The promoter is dialysable. This indicates that its molecular weight is probably less than 600. If we assume the molecular weight
is about 400, then 2 grms in 5 ml of distilled water would be a molar solution. 17 mgrms of material were eluted from the major promotive area of the paper chromatogram and dissolved in 5 ml of distilled water. Assuming a molecular weight of 400, this represents a $8.5 \times 10^{-3}$ molar solution. It was shown that when a ten dilution was carried out of this eluted material the optimum germination was at a $10^{-1}$ dilution of the eluate, i.e., at a molar concentration of $8.5 \times 10^{-4}$ based on the above assumptions. It was shown in the thin layer chromatograms following that this eluted material was still relatively impure. Half this eluted material was used to obtain the eluate used in Expts 33a and 34a, where the optimum germination was achieved when the eluate of the thin layer chromatograms was diluted to $10^{-2}$. This corresponds to a concentration based on the above assumptions of less than $4.2 \times 10^{-5}$ molar.
CHAPTER V

Some Factors Affecting the Infection of the Leaves of Certain Species of Eucalyptus by Phaeoseptoria eucalypti

5.1 Introduction

The relationship between susceptibility to infection and susceptibility to disease, is often not clear in the literature of plant pathology. Some of the confusion arises from the difficulty of precise definition of the terms "disease" and "infection" and some stems from the false synonymy in early literature of disease and pathogen (Horsfall, 1960). Athosporium victoriae on oats (Paddock, 1953) and Venturia inaequalis appear on White's classification it is possible to recognise resistance in plants in the following classes, (Muller, 1958; Cruickshank, 1963). In some instances hypersensitive Infection reactions and Phytoalexin Disease Resistant (unreactive plants) in the Physiological Resistant Susceptible (reactive plants) are regarded as part of the infection, not post infection.
This classification because of its simplicity and clarity has served a very useful purpose. Certain developments and interpretations have however made the distinction between these groups less precise.

The major difficulty which arises results from the definition of infection. The Axeny of Gaumann (1950) is regarded as the classical example of unreactive plants e.g. protocatechuic acid in the pigmented scales of onions inhibiting infection by Colletotrichum circinans (Walker, 1923). The hypersensitive necrogenic reactions of resistant hosts to obligate parasites could be regarded on White's (1957) classification as typical of plants susceptible to infection but resistant to disease.

Investigations have shown that hypersensitive necrogenic reactions are not restricted to obligate parasites e.g. Helminthosporium victoriae on oats (Paddock, 1953) and Venturia inequalis on apples (Nusbaum and Keitt, 1938). It would appear that hypersensitive necrogenic reactions may be only one expression of the phenomenon covered by the phytoalexin concept (Muller, 1958; Cruickshank, 1963). In some instances hypersensitive necrogenic reactions and phytoalexin production reactions have been discussed in the physiology of penetration and infection (Flentje, 1959) i.e. these reactions are regarded as part of the infection, not post infection, phenomena.
1.

This latter classification is accepted then the distinction between resistance to infection and susceptibility to infection, but resistance to disease, becomes merely a matter of when the host reaction to the pathogen occurs. The formation of resin plugs in the roots of *Pinus* as a reaction to infection by *Fomes annosus* (Risbeth, 1951) would presumably be a post-infection reaction because it occurs long after the initial infection has been established.

It also seems likely that many plants which would be regarded under White's classification as non-reactive, i.e., wrong hosts are those whose phytoalexin production, following inoculation by the pathogen, is sufficiently high to inhibit the growth of the particular pathogen. In such cases there is no histological evidence of infection and no parasitic relationship has been established with the host but the pathogen is inhibited.

Further difficulties arise when it is appreciated that the barriers to infection are modified by environment. (Wingard, 1941). In this context a plant has the genetic capacity to produce a barrier to infection but the extent to which the barrier is produced or is effective depends on the environment. The distinction between this type of resistance and disease escape is not particularly evident. The concept of inoculum potential is worthy of mention in this regard.
A plant may show resistance to infection, not because a barrier affects the infection process, but because the barrier prevents the spore load reaching the threshold level for a single infection to occur. Such a plant has a barrier but it is in reality a barrier to inoculation and it would be more correct to say the plant escapes inoculation and consequently escapes disease. In the same way, a particular grain crop variety escapes disease in the relative absence of the pathogenic strains adapted to it.

In the experiments which follow, infection is used in the sense of a prepenetration phenomenon. The investigation has concentrated on Axeny type factors present in the host regardless of the presence of the pathogen. It is possible that resistance to the disease caused by Phaeoseptoria eucalypti in Eucalyptus may be determined more by reactions of the host, as a result of infection by the pathogen, but this form of resistance has not been investigated.

5.2 Objectives of the investigation

In the Botany Department glasshouses it was observed that the juvenile glaucous leaves of E. bicostata were only lightly infected by Phaeoseptoria eucalypti. Leaves of E. tereticornis, E. grandis, E. saligna and other relatively non-glaucous species showed a heavy infection by the pathogen when exposed in the same environment. When E. bicostata
juvenile foliage was artificially inoculated with a heavy spore suspension, particularly if the leaves were lightly rubbed between the fingers or wiped with cotton wool before inoculation, a very heavy infection was obtained. These observations suggest that _E. bicostata_ juvenile leaves have some degree of resistance to the disease produced by _P. eucalypti_. The experiments described aimed at accounting for at least some of these obvious differences in susceptibility, between species of the subgenus Macranthera (Pryor, 1959), and within a species, according to inoculation and conditions.

5.3 Experiment No. 18 Effect of wiping leaves of _E. bicostata_ raised in the glasshouse on infection by _P. eucalypti_. Twelve plants of _E. bicostata_ grown in the Botany Dept. glasshouse were taken and the tops removed to reduce each plant to 3 pairs of opposite leaves. The leaf pairs on the different plants were all of approximately the same age. Each plant was taken and one member of each leaf pair was the lightly wiped with cotton wool to remove the superficial glaucous coating. Observations have indicated that the glaucous coating on eucalypt leaves can be almost quantitatively removed by wiping with cotton wool (Lamberton, 1964). The leaves for wiping were selected in a right hand spiral up the plant from the lowest leaf pair.
After wiping the leaves were inoculated by placing the pots containing the plants, in three groups of four plants each, on a rotating table. As the table rotated the plants were sprayed with a heavy spore suspension from a spore store attached to a compressed air line. To ensure a uniform spore load in the spraying of successive groups of plants the spray was turned on for a fixed period—while the table was rotating. During each group spraying two glass slides, held horizontally, were exposed at the level of the plant leaves. At the conclusion of each group spraying these were removed, marked and dried. The spore numbers per unit area on the glass slides for each spraying were later assessed. The spore numbers per unit area of slide did not differ significantly between the different plant groups sprayed.

The plants were incubated for 48 hours in a glass fronted incubator at 25°C. Additional light was supplied from 2x150 watt Condor lamps. (See Chapter 1) The humidity was maintained at a high level by a shallow tray of water in the base of the incubator.

After incubation the plants were placed on the benches in a glasshouse well removed from any other plants infected with *P. eucalypti*. After 30 days chlorotic areas appeared on the infected leaves and at 40 days the lesions were reddish brown and fructifications of the pathogen were numerous.
TABLE 5. Percentage of leaf area lesioned on leaves of *E. bicostata* seedlings in relation to the wiping treatment and to the nodal position of the leaves on the plants (12 plants, 3 pairs of leaves on each plant, with one member of each leaf pair wiped, used in the experiment).

<table>
<thead>
<tr>
<th>Leaf Position</th>
<th>Wiping Treatment</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wiped</td>
<td>Unwiped</td>
<td></td>
</tr>
<tr>
<td>Lowest Leaf Pair</td>
<td>14.09</td>
<td>4.56</td>
<td></td>
</tr>
<tr>
<td>2nd Lowest Leaf Pair</td>
<td>9.61</td>
<td>2.47</td>
<td></td>
</tr>
<tr>
<td>3rd Lowest Leaf Pair</td>
<td>11.58</td>
<td>5.22</td>
<td></td>
</tr>
</tbody>
</table>
At this stage the individual leaves were removed from the plants. Areas were determined, from the traced outline of leaves, by a planimeter. The lesioned area was assessed by using a transparent sheet of plastic marked in 0.25 cm squares. The percentage of the total leaf area lesioned was then calculated from this data for each leaf. The data for the various leaves was summarised according to the leaf position on the plant and the wiping treatment applied. The mean percentage leaf area lesioned for each leaf position group, and wiping treatment was calculated. The statistical significance of the observed differences were calculated using a paired T-test.

The results are presented in Table 5.

Discussion

1. The wiping of one member of the lowest leaf pair on each plant results in an increase of 208% (Sig. at P < .001) in the mean percentage of the total area of those leaves lesioned by comparison with the opposite unwiped leaves.

2. The wiping of one member of the second lowest leaf pair on each plant results in a mean increase of 289% (Sig. at P < .01) in the mean percentage of the total area of those leaves lesioned by comparison with the opposite unwiped leaves.

3. The wiping of one member of the third lowest leaf pair on
each plant results in an increase of 121% (N/S at P = .05) in the mean percentage of the total area of those leaves lesioned by comparison with the opposite unwiped leaves.

The above results indicate the considerable increase in lesioned area which results from the removal of superficial "wax" from the 2 lowest pairs of leaves before inoculation. In the case of the third pair of leaves although the mean lesioned area also increased with wax removal, the increase was not significant because of the high variability in percentage area lesioned on different plants.

A cursory examination of the plants also seemed to show a general trend for decreased lesioned area on leaves from the lowest to the top most leaf pair, in unwiped leaves i.e. the oldest leaves on each plant appear to be the most susceptible to the disease. The data were analysed using Student's T test. This trend was not significant at the P = .05.

It was decided to carry out a further experiment of the relationship between lesioned area, leaf position and wiping treatment. The plants used had been raised in the high nutrient, more uniform environment of the Ceres phytotron. In this experiment it was also decided to use leaves whose position was more separated than those used in Expt 18.

5. 4 Experiment No. 19 Effect of wiping leaves and leaf position on E. bicostata plants, raised in the
phytotron, on infection by *P. eucalypti*

For the purpose of this experiment seeds of *E. bicostata* were germinated on moist filter paper over vermiculite in petri dishes in the Ceres phytotron. At 2 weeks, when the seedlings had well developed radicles and cotyledons, the seedlings were placed in 5" plastic pots of perlite. Initially 3 seedlings were placed in each pot. These were thinned to one, well developed seedling at 1 month from germination. The *E. bicostata* seedlings were raised in the 27°C phototemperature 21°C nyctotemperature glasshouse of the phytotron. In the glasshouse the seedlings had a light regime of 8 hours dark, 8 hours photosynthesis and 16 hours photoperiod. They were watered with half strength, Hoaglands nutrient solution each morning and demineralised water each afternoon. At two months four uniform well developed plants, each with about thirteen pairs of opposite leaves above the cotyledons were selected.

The apical bud and the three top pairs of leaves of each plant were removed. The plants were then treated as illustrated in Diag 5/1. It was not possible to leave exactly comparable aged leaves (in relation to the apical bud) on each plant because occasional leaves were damaged or abnormal in some respect and these were cut off.

After the leaf pruning described the plants were taken and one leaf of each pair, on each plant, was lightly wiped
Leaf pruning treatment applied to *E. bicostata* plants before inoculation.
### TABLE 5.4.1 Number of Lesions produced on the leaves of E. bicostata by P. eucalypti with different wiping treatments.

<table>
<thead>
<tr>
<th>Wiping Treatment</th>
<th>Wiped Leaves</th>
<th>Unwiped Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Lesion number</td>
<td>239</td>
<td>48</td>
</tr>
<tr>
<td>Total Leaf area (sq. cms)</td>
<td>950.16</td>
<td>1086.52</td>
</tr>
<tr>
<td>Lesion number per sqr cm</td>
<td>0.251</td>
<td>0.044</td>
</tr>
</tbody>
</table>

These results were then tested for significance by an Analysis of Variance Test presented in Table 5.4.3.

### TABLE 5.4.2 Number of Lesions produced on the leaves of E. bicostata by P. eucalypti in relation to leaf position.

<table>
<thead>
<tr>
<th>Leaf Position</th>
<th>Upper Leaves</th>
<th>Lower Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion number per sqr cm</td>
<td>1.045</td>
<td>1.892</td>
</tr>
</tbody>
</table>
with cotton wool. The wiping treatment was applied to leaves selected in a right hand spiral up the plant. The plants were inoculated with a heavy spore suspension of *P. eucalypti* and incubated for 48 hours as described in Expt 18.

After the incubation period the plants were placed in a growth cabinet in the Botany Dept. This cabinet had a light regime of 8 hours dark, 8 hours fluorescent lighting and 8 hours incandescent lighting. The cabinet had a photo temperature of 27°C and nycotemperature of 22°C. The plants were watered with half strength Hoagland solution each morning and demineralised water each afternoon.

At 32 days lesions were apparent on the leaves and fruits of *P. eucalypti* were sporulating on the lesions.

The leaves were removed individually from the plants, the outline of each leaf traced on paper and its area assessed with a planimeter. The number of individual lesions on each leaf was recorded. The lesion number per unit area was computed. The data were then summarised into lesion number per unit area for the wiping treatments. The data for all leaves were also summarised into lesion number per unit area, for the two levels i.e. the upper leaf and lower leaf groups.

The results are presented in Table 5.41 and 5.42.

**Discussion**

From the above experiment it can be concluded that
### TABLE 5.4 3 Analysis of Variance Table

<table>
<thead>
<tr>
<th>Variation due to</th>
<th>Degrees of Freedom</th>
<th>Mean Sum of Squares</th>
<th>Variance Ratio</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wiping</td>
<td>1</td>
<td>.487</td>
<td>37.045</td>
<td>Sig @ P .001</td>
</tr>
<tr>
<td>Levels</td>
<td>1</td>
<td>.086</td>
<td>6.542</td>
<td>Sig @ P .05</td>
</tr>
<tr>
<td>Plants</td>
<td>3</td>
<td>.017</td>
<td>1.315</td>
<td>N/S</td>
</tr>
<tr>
<td>Wiping x Level interaction</td>
<td>3</td>
<td>.002</td>
<td>.154</td>
<td>N/S</td>
</tr>
<tr>
<td>Error</td>
<td>39</td>
<td>.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Assessed from Variance Ratio Table (Fisher and Yates, 1948).
1. The light wiping of the juvenile leaves of *E. bicostata* with cotton wool to remove surficial glaucousness, increases the lesion number per unit area by 470% (Sig P<.001) by comparison with comparable unwiped leaves.

2. The lesion number per unit area on the three lower leaf pairs is greater than that on the three upper leaf pairs by 81% (Sig at P<.05).

3. There is no significant difference between lesion numbers per unit area on different plants used in the experiment.

4. There is no significant interaction between wiping and leaf level in this experiment.

**Conclusion** (1) above is in agreement with the result of Expt 18. Taken together these experiments indicate that, independent of the cultural environmental conditions under which the plants are raised and maintained in the experiments, the superficial glaucous covering on the juvenile leaves of *E. bicostata* is a significant factor in determining the degree of disease development achieved on these leaves by *P. eucalypti*. In the first experiment the lesioned area was taken as the index of infection. In this experiment the number of primary lesions per unit leaf area was taken as the index. With *P. eucalypti* the spread of an individual lesion is limited by the veins surrounding the area of infection. It is to be expected that the use of either index will yield substantially
the same result.

It may be objected that in the light wiping by cotton wool to remove the glaucous layer the leaf is mechanically wounded and that the increased infection following wiping result from this wounding and not from the removal of the glaucous layer.

Electron microscope photographs of carbon replicas of wiped and unwiped leaves show no evidence of mechanical damage to the surface by light wiping (See Plates 5/1 and 5/2). The cuticle of these juvenile leaves is probably resistant to light abrasion. In Expt 23 of this series E. bicostata plants were grown at different temperatures in the Ceres phytotron. These temperatures resulted in different quantities of ether solubles on the surface of the leaves. The degree of disease development following inoculation of these plants, grown at different temperatures, shows a good but not significant negative correlation with the weight of ether solubles per unit area of leaf. This would further support the contention that it is the glaucous covering itself which affects the degree of infection of the leaf by P. eucalypti.

If it is assumed that the presence of the glaucous layer is itself significant in the degree of leaf infection by P. eucalypti, how could the layer affect the degree of infection and what experiments could be designed to illustrate
Electron microscope photograph of carbon replica of the surface of *E. bicostata* leaf grown at 27°C phototemperature and 22°C nycotemperature before wiping to remove superficial glaucousness.

Magnification on negative 1600 x
As for Plate 5/1 but after light wiping with cotton
wool to remove superficial wax note there is no evidence
of damage to the leaf surface although the coating of wax
has been largely removed.

Magnification on negative 1600 x
that the layer is operative in the postulated way?

a. The layer, because of its waxy nature, affects the wettability of leaves. The number of spores deposited from a spore solution on a wiped leaf could consequently be greater than on an unwiped leaf from the same spore solution. This suggestion was tested in the series of experiments on spore deposition.

b. The glaucous layer could contain materials which affect the production and growth of germ tubes of spores deposited on it. It has been shown in Expt 16 that the water soluble fraction of the ether solubles from the surface of juvenile leaves of E. bicostata, at the concentrations used in the experiment, contained materials inhibitory to the germination of the spores of P. eucalypti. The inhibition of germination by these materials produced was significant but not very great and probably insufficient to account for the above observed great differences in disease intensity between wiped and unwiped leaves. No test has been carried out of the significance of the glaucous material in germ tube growth.

c. The glaucous layer could have a mechanical effect on the growth and stomatal penetration of the germ tube. No experiments have been conducted to test this possibility. It has been observed however that closed stomata do not prevent...
penetration. Juvenile leaves kept in the dark are just as severely penetrated by germ tubes of the fungus, through the stomata, as are leaves kept in the light.

All the above suggestions as to the possible mechanism of resistance of the glaucous barrier have assumed that the resistance of glaucous leaves is of the Axeny type (Gaumann, 1950) i.e. it is passive resistance dependent on a barrier present in the host irrespective of the presence of the fungal pathogen. It is also not pathogen specific, it may be more effective against some pathogens than others but its general defensive nature is independent of the pathogen. The assumption that resistance in this instance depends on Axeny type factors maybe unjustified (see Expt 2).

Conclusion (2) of the above experiment showing a significantly higher infection of lower by comparison with upper group of leaves, is in general agreement with the results for the relative susceptibility of leaves up the tobacco plant to infection by *Peronospora tabacina*, where primary lesion number per unit area is the index used for assessment of disease (Hill, 1959). It has been shown recently that there is a good correlation, in the case of *Nicotiana debneyi* C.V. 120, of the number of inhibitor units per sq cm of leaf surface and the susceptibility of leaves at successive levels to infection. This correlation has suggested to the authors
that the number of inhibitor units per sq cm of leaf surface at successive levels may be the explanation for the observed decrease in susceptibility of successive leaves up the plant (Shepherd and Mandryk, 1963). The situation could be essentially the same with P. eucalypti on the juveniles of E. bicostata. The amount of glaucous covering being the significant factor in this case. This has been the subject of a later experiment. There are however a number of other possible explanations for this observation, e.g.:

a. The lower leaves maybe a more satisfactory substrate for the organism than the upper leaves. If leaves were left unwiped, age maybe a factor in susceptibility of leaves to disease. In the case of Phytophthora cactorum causing die-back of rhododendrons, in the bud and bud scales, leaves can be successfully inoculated. Leaves one year or older are resistant (Pirone, 1940). The lower leaves are not only physiologically older, they were produced at a different ontogenetic stage in the plant's life. This could be the explanation of the difference in susceptibility. The mean % area c. The lower leaves may have a different active resistance level from the upper leaves. This possibility can only be investigated when differences in resistance of the Axen type have been eliminated.

A further experiment was carried out on infection in lesioned area than the 7th node leaf pair.
in relation to leaf level to see if significant differences in infection could be obtained between adjacent leaf pairs up the plant before proceeding with an investigation of some of these possibilities.

5.5 Experiment 20 Relative susceptibility to infection by _P. eucalypti_, of adjacent leaf pairs of _E. bicostata_

In this experiment seven plants of _E. bicostata_ were raised in the Ceres phytotron as described for Expt 19, when 2 months old the tops of the plants were cut off to reduce them to 3 pairs of opposite leaves, the 5th, 6th and 7th node above the cotyledons, on each plant. All leaves were left unwiped and the plants were inoculated, incubated and kept in the growth cabinet as described in Expt 19. In the growth cabinet the plants were pruned of axillary buds. After 40 days when the lesions with well developed and fructifications were developing the individual leaves were removed and their area assessed by planimeter. The area lesioned was computed as before using a clear .25 cm square grid. The percentage area lesioned for each leaf was calculated and the mean % area lesioned for each leaf position was computed. The results are presented in Table 5.5.1.

The 5th node leaf pair has 191% more lesioned area than the 6th node leaf pair and 877% more lesioned area than the 7th node leaf pair. The 6th node leaf pair has 236% more lesioned area than the 7th node leaf pair.
TABLE 5.1 Percentage area of *E. bicostata* leaves, at different positions up the plant, lesioned by *P. eucalypti*.

<table>
<thead>
<tr>
<th>Leaf position above cotyledons</th>
<th>Mean percentage leaf area lesioned</th>
</tr>
</thead>
<tbody>
<tr>
<td>5th node</td>
<td>36.48</td>
</tr>
<tr>
<td>6th node</td>
<td>12.51</td>
</tr>
<tr>
<td>7th node</td>
<td>3.73</td>
</tr>
</tbody>
</table>

TABLE 5.2 Analysis of Variance Table

<table>
<thead>
<tr>
<th>Variation due to Levels (1, 2 and 3)</th>
<th>Degrees of freedom</th>
<th>Mean Sum of Squares</th>
<th>Variance Ratio</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levels (1, 2 and 3)</td>
<td>2</td>
<td>2012.48</td>
<td>25.71</td>
<td>Sig @ P&lt;.001</td>
</tr>
<tr>
<td>Within Levels</td>
<td>18</td>
<td>78.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Assessed from Variance ratio tables (Fisher and Yates, 1948).
The results were analysed using an Analysis of Variance test, presented in Table 5.5.2. The analysis indicates that the level of the leaf in the plant is a highly significant factor in the degree of infection of the leaf by \( P. \) eucalypti. To establish whether the leaf level is significant between adjoining leaf pairs, a test must be carried out using the calculated mean infection level for adjoining leaf pairs. For this purpose, the Mean Sum of Squares Within Levels variation in Table 5.5.2 is the best value for calculation of the standard error of the mean.

\[
S.E. \text{ of the mean } = \sqrt{\frac{78.28 \times 2}{3}} = \sqrt{\frac{156.56}{3}} = \sqrt{7.21} = 1.56\bar{5}
\]

This difference of 23.7% is not significant at the \( p = 0.05 \). In test for significance, \( T = \frac{\text{Difference of Means (D)}}{S.E.} \)

\[
\text{Difference of means } = S.E. \times T
\]

From a Table of Values of Student's \( T \) (Fisher and Yates, 1948), the value of \( T \) for significance with 18 degrees of freedom at the 5% level = 2.101, at the 1% level = 2.878 and at .1% level = 3.923. Thus for the difference of means of different particular levels to be significant at these levels, the following is accessory.

For \( P = or< .05 \) difference of means must = or exceed \( 7.21 \times 2.101 \text{ i.e. } 15.14 \)

For \( P = or< .01 \) difference of means must = or exceed \( 7.21 \times 2.878 \text{ i.e. } 20.750 \)
For \( P = \text{or} < .001 \) difference of means must = or exceed 7.21 x
3.922 i.e. 28.277

If these criteria are applied to the particular levels:

5th node Leaf Pair / 6th node Leaf Pair:
Diff. of means of level of infection = 23.97. This
is a difference of 193% Sig. at \( P < .01 \).

5th node Leaf Pair / 7th node Leaf Pair:
Diff. of means of level of infection = 32.75. This
is a difference of 878% Sig. at \( P < .001 \).

6th node Leaf Pair / 7th node Leaf Pair:
Diff. of means of level of infection = 8.78. This
difference of 235% is not significant at the \( P = .05 \).

The failure of the difference of means of level of infection
between 6th node and 7th node Leaf Pair to reach significance
using the Standard Error calculated from the Analysis of
Variance Table is because this figure is inflated by the
great variation of infection level in the 5th node Leaf pair.
It can be shown by a Variance ratio test that the populations
of the 5th node and 6th node leaf pairs are not homogenous.
On the other hand a similar test using the populations of the
6th and 7th node Leaf Pairs shows that these populations are
homogenous. It is hence satisfactory to assess the signific-
ance of the difference between the mean level of infection of
these two groups of leaves on Student's T test using a figure
of Standard Error calculated from the Variances of these two populations only i.e. excluding the variation due to the lowest leaf pair inherent in the above computed Standard Error. On this basis the difference of means of level of infection of the 6th node leaf pair and the 7th node leaf pair of 23.5% is significant at P < 0.01.

Discussion

These results confirm those of the previous experiment on the significance of leaf level in the degree of infection of the juvenile leaves of *E. bicostata* by *P. eucalypti*. They also show that even adjoining leaf pairs show considerable, significantly different, infection levels. The result is in contrast with observations of glume blotch of wheat, caused by *Septoria nodorum* (Scharen, 1963). Here, using detached leaves, the degree of infection of wheat leaves is independent of leaf age. The results are also in contrast to those for *Septoria passerinii* on barley where younger leaves are more susceptible than older leaves to infection by the pathogen (Green and Dickson, 1957). The results are in agreement with observations on the susceptibility of tobacco leaves at different levels on the plant to infection by *Peronospora tabacini* (Hill, 1959). The results of the experiments conducted, show that with the glaucous juvenile foliage of *E. bicostata*
1. The wiping of leaves, at all levels on the plants used in these experiments, has the effect of significantly increasing the susceptibility of these leaves to the leaf spot caused by *P. eucalypti*.

2. That independent of wiping, the leaves at different levels on the plant differ in their susceptibility to this disease even if the particular leaves compared, differ by only one plastochron in ontogenetic age.

Some of the possible explanations for these two general conclusions have already been advanced. The following experiments are concerned with testing the significance of some of these explanations.

5. **Experiment 21** Effect of lightly wiping the glaucous juvenile leaves of *E. bicostata* on the deposition of spores from a spore suspension of *P. eucalypti*.

Among the possible explanations of the effect of wiping on the disease susceptibility of *E. bicostata* leaves was the suggestion that the presence of the superficial glaucous layer affects the wettability of the leaf surface and consequently the adherence of droplets and the deposition of spores. It is also possible that the deposition of spores on unwiped leaves at different levels on the plant is significantly variable. These two factors i.e. wiping and leaf level were tested in the following way.
Four plants of *E. bicostata*, carrying juvenile leaves only, were taken. These plants were reduced to six pairs of leaves (3 pairs at nodes 3–5 and 3 pairs at nodes 8–10). One member of each leaf pair on each plant was wiped lightly with cotton wool. The member of each leaf pair selected for wiping was on the basis of a right hand spiral up the plant from the base. The plants were sprayed as described previously with a heavy, uniform suspension of *P. eucalypti* spores. After spraying the plants were left standing in a still environment for 4 hours to allow the spore suspension to dry on the leaves.

When the leaves were dry, each leaf was individually taken from the plant and marked. The leaf was divided into approximately three equal sections along the length of the midrib and the central portion across the leaf was retained. This section was cut down the middle and the midrib removed. Each leaf portion was cleared and stained following the procedure outlined in Chapter 3 for similar leaf pieces. Each leaf piece was then mounted on a microscope slide, underside uppermost, in lacto-phenol and covered with a coverslip.

The number of spores deposited was assessed by counting the number of spores per Low Power Field of a Zeiss Binocular Microscope in 45 fields on each leaf piece i.e. 90 fields per leaf. The mean number of spores per Low Power Field of each leaf was calculated.
TABLE 5.61 The effect lightly wiping E. bicostata juvenile leaves on the deposition of spores of P. eucalypti from a spore suspension.

<table>
<thead>
<tr>
<th>Wiping treatment</th>
<th>Leaf wiped</th>
<th>Leaf unwiped</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of Spores deposited on 90 L.P. fields</td>
<td>729</td>
<td>518</td>
</tr>
</tbody>
</table>

The above means differ by 1307%.

TABLE 5.62 The effect of the level of E. bicostata juvenile leaves on the deposition of spores of P. eucalypti.

(Using unwiped leaves only).

<table>
<thead>
<tr>
<th>Leaf Position</th>
<th>Lower Leaves</th>
<th>Upper Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of spores deposited on 90 L.P. fields</td>
<td>70</td>
<td>24.8</td>
</tr>
</tbody>
</table>

These means differ by 182%.
When the count on the first plant had been completed it was apparent the effect of wiping was very pronounced. A comparison between wiped and unwiped leaves was made for this plant only. (Inspection of random pieces from the other plants showed the same general pattern). The effect of leaf level was not so obvious. A count was made for the mean number of spores deposited per Low Power Field on the unwiped leaves of all plants.

The results are presented in Tables 5.6.1 and 5.6.2.

A variance ratio was computed for the two treatments to establish if the populations were homogenous and if their variances could be pooled. The Variance Ratio of 77.739 is significant at the $P = .05$ level. A Behren's modified T test was used to permit a comparison being made between two populations whose variances are not comparable (Fisher, 1946). Using this test it was shown that the very considerable difference between spore deposition on wiped as against unwiped leaves is significant at $P < .01$.

The variance ratio computed from the variances of the two populations was significant at $P = .05$ level. An Analysis of Variance Test was used to compute the significance of the difference of the means.

This is presented in Table 5.6.3.
<table>
<thead>
<tr>
<th>Variation due to</th>
<th>Degrees of Freedom</th>
<th>Mean Sum of Squares</th>
<th>Variance Ratio</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levels</td>
<td>1</td>
<td>12,285</td>
<td>8.793</td>
<td>Sig @ P&lt;.01</td>
</tr>
<tr>
<td>Plants</td>
<td>3</td>
<td>1,695</td>
<td>1.213</td>
<td>N/S @ P=.05</td>
</tr>
<tr>
<td>Error</td>
<td>19</td>
<td>1,397</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Assessed from Variance Ratio Tables (Fisher and Yates, 1948)*
Discussion

Conclusions which can be drawn from this experiment are:

1. The spore load of *P. eucalypti* deposited from a heavy spore solution on wiped juvenile leaves of *E. bicostata* is 1307% (Sig at P<.01) higher than that deposited on comparable unwiped glaucous juvenile leaves on the same plant.

2. The spore load of *P. eucalypti* deposited from a heavy spore solution on groups of three lower wiped leaves of *E. bicostata* is 187% greater, (Sig at P<.01), than that deposited on groups of three upper unwiped leaves of the same plants.

3. There was no significant difference between the spore drop loads deposited on groups of upper or lower leaves on different plants in this experiment. (Durchfield and Goema).

These results provide a very obvious explanation for the considerable differences in lesioned area (or lesion number) observed in the comparison on wiped and unwiped fungal leaves in Expts 18 and 19. The observations indicate that the presence of the superficial glaucous material on leaves has a considerable effect on the number of spores deposited per unit area of leaf, probably per medium of the effect of the layer on wettabillity. Account must be taken of the glaucous
contention that in general the **Inoculum Potential** of a single spore varies according to whether the spore is of biotroph or a necrotroph (White, 1957). From the viewpoint of the infecting fungus the presence of a glaucous layer on the juvenile leaves of *E. bicostata* reduces the **Inoculum Potential** of the spore load, which is sprayed or splashed (in nature) at it. It can be expected that the disease intensity will be affected by this change in **Inoculum Potential**.

The above observations on the significance of wettability of leaves in the deposition of spores from solution are in agreement with those on the significance of wettability in the deposition of spray droplets of fungicides, (Burchfield, 1959). The importance of the waxy blooms on banana leaves in the deposition of Bordeaux mixture sprays, whose droplets "hit and bounced off such leaves as if they were minute rubber balls" has been discussed, (Burchfield and Goenaga, 1957).

In addition to the effect of the glaucous layer as an anti-wetting agent, account must also be taken of its fungicidal properties illustrated by its effect, at particular concentrations on the germination of the spores of *P. eucalypti*. (Expt 16)

Qu. 7 Quite obviously from these observations if there is "active resistance" of glaucous by comparison with non-glaucous
leaves, this phenomenon will only show up experimentally if the experimental arrangement is such as to eliminate these two Axeny type resistance mechanisms.

The experimental results also show that there is a significantly higher spore deposition on lower by comparison with upper leaves. This observation parallels the observation on the levels of diseased area or lesion number per unit area on these groups of leaves. It is reasonable to postulate that the higher deposition of spores on lower leaves by comparison with upper leaves is a significant factor in the higher incidence of diseased area or lesion number per unit area on the lower by comparison with the upper leaves. The observation on deposition is in agreement with results of studies on the accumulation of copper from Bordeaux mixture sprays on the surface of old by comparison with young banana leaves (Burchfield and Goenaga, 1957). This observation however offers no explanation as to why the deposition of spores should be heavier on lower than on upper leaves. In some of the experiments which follow, an attempt is made to explain this observation and also to investigate more fully the relative resistance of upper and lower leaves to the disease.

5. 7 Experiment 22 The effect of lightly wiping the leaves of, and the significance of leaf position in, E.
### TABLE 5.7.1 Effect of lightly wiping the juvenile leaves of *E. tereticornis* on spore deposition from *P. eucalypti* spore suspension.

<table>
<thead>
<tr>
<th>Wiping Treatment</th>
<th>Wiped Leaves</th>
<th>Unwiped Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Number of spores deposited on 90 L.P. fields</td>
<td>208.9</td>
<td>47.1</td>
</tr>
</tbody>
</table>

The above means differ by 34.3%.

### TABLE 5.7.2 Effect of level of leaf on the plant of *E. tereticornis* on deposition of spores of *P. eucalypti* from a spore suspension.

(Unwiped leaves only).

<table>
<thead>
<tr>
<th>Level of Leaf on Plant</th>
<th>Upper Leaves</th>
<th>Lower Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean number of spores deposited on 90 L.P. fields</td>
<td>70.0</td>
<td>24.3</td>
</tr>
</tbody>
</table>

The above means differ by 188%.
tereticornis seedlings on the deposition of spores from a spore suspension of P. eucalypti.

In the glass house it was observed that on the relatively non-glaucous E. tereticornis juveniles (they show a slightly waxy appearance only) the pattern of lesioned area, or lesion number unit area up the plant was similar to that described for E. bicostata juveniles. It was decided to carry out a spore deposition experiment on this species to examine the effect of the wiping of leaves and also leaf ability and this layer can be removed by light wiping with level on spore deposition. A single plant of E. tereticornis grown in the Ceres phytotron was selected, Groups of 4 lower leaves in the two experiments indicates that the glaucous and 4 upper leaf pairs (the juveniles are not quite opposite layer of E. bicostata is each more effective (nearly four times), than the non-glaucous layer of E. tereticornis in E. bicostata. The spraying with spore solution, drying, this as a firm quantitative conclusion since, in these experiments, although a number of leaves are involved, only one used in the previous experiments.

The results are presented in Table 5.7.1.

It is reasonable, in view of the great difference shown, to conclude that the glaucous layer is more efficient than the non-glaucous in reducing the spore deposition of P. eucalypti. Although in this case only one plant was used the of P. eucalypti on wiped juvenile leaves of E. tereticornis pattern of spore deposition in relation to leaf level is the is 34.3% (Sig @ P < .001) higher than on comparable unwiped same as for E. bicostata i.e. there is a significantly higher juvenile leaves of the same plant. spore deposition on lower than on upper unwiped leaves.

Discussion

From these results it can be concluded that a. The spore load deposited from a heavy spore solution
b. The spore load deposited from a heavy spore solution on a group of 4 lower unwiped leaves of *E. tereticornis* is 187% (Sig @ P < .01) higher than that deposited on a group of 4 upper unwiped leaves of the same plant.

These results for *E. tereticornis* are in agreement with those for *E. bicostata* in the previous experiment. Apparently even on the non-glaucous leaves of *E. tereticornis*, there is a superficial layer of material which probably reduces wettability and this layer can be removed by light wiping with cotton wool. A comparison of the relative effect of wiping leaves in the two experiments indicates that the glaucous layer of *E. bicostata* is much more effective (nearly four times), than the non-glaucous layer of *E. tereticornis* in reducing spore deposition. It is probably not safe to make this as a firm quantitative conclusion since, in these experiments, although a number of leaves are involved, only one plant of each species has been tested for the effect of wiping.

It is reasonable, in view of the great difference shown, to conclude that the glaucous layer is more efficient than the non-glaucous in reducing the spore deposition of *P. eucalypti*.

Although in this case only one plant was used the pattern of spore deposition in relation to leaf level is the same as for *E. bicostata* i.e., there is a significantly higher spore deposition on lower than on upper unwiped leaves.
(The close agreement between the degrees of difference, 187%, in both *E. tereticornis* and *E. bicostata*, may be purely fortuitous or may indicate that a similar or the same factor is operative in both species).

In the experiments performed so far on the effect of wiping juvenile leaves, on diseased area or lesion number per unit area, the comparison has been between fully glaucous leaves and leaves from which, for all practical purposes, the glaucousness has been completely removed (compare Electron Microscope Photographs Plates 5/1 and 5/2). If the effect of glaucousness is as considerable as indicated by these experiments, it might be possible to obtain a correlation between degrees of glaucousness and degrees of disease area or lesion number per unit area. Various degrees of glaucousness could probably be obtained by raising plants in the Ceres phytotron at different temperatures (Whitecross, 1963; Daly, 1964).

5.8 Experiment 23 Effect of temperature of growth on the glaucousness of *E. bicostata* leaves and the relationship of this to susceptibility to infection by *P. eucalypti*.

For this experiment seeds of *E. bicostata* were germinated in the usual way. Three seedlings were pricked out into each of 60 plastic pots containing perlite. The 60 pots were divided into 3 groups and one group of pots was placed in
each of the glasshouses of the phytotron with the following temperature conditions.

<table>
<thead>
<tr>
<th>Glasshouse</th>
<th>Photo Temperature</th>
<th>Nycotemperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27°C</td>
<td>22°C</td>
</tr>
<tr>
<td>2</td>
<td>21°C</td>
<td>16°C</td>
</tr>
<tr>
<td>3</td>
<td>15°C</td>
<td>10°C</td>
</tr>
</tbody>
</table>

At three weeks the seedlings were thinned to 1 per pot. At ten weeks the plants grown in the 21°C and 27°C glasshouses had 8 pairs of fully expanded juvenile leaves. The plants in the 15°C house had only 3 pairs of fully expanded leaves and the growth of these plants was very slow. This is in agreement with other observations on the species (Scurfield, 1961). These latter plants would take a very long while to form 8 pairs of opposite leaves by which time the plants in other higher temperature houses would be too large for use in the experiment. It was decided to use all plants at this 10 week stage for harvesting and for inoculation.

15 plants of each temperature treatment were taken and divided into 3 groups of 5 plants each. These plants were to be used for assessment of "glaucousness per unit leaf area". Three glass, 200 cc, beakers were taken and 100 cc of ether placed in each beaker. The first group of 5 plants of the 27°C plants was taken. Leaves of the 1st, 2nd and 3rd leaf pair above the cotyledons were removed. The length and
TABLE 5. 8 1 Weight of Ether solubles per unit leaf area of \textit{E. bicostata} plants grown at different temperatures.

(3 replicates each of 5 plants for each temperature treatment).

<table>
<thead>
<tr>
<th>Day Temp. at which plants grown</th>
<th>Replicate</th>
<th>Leaf Position on Plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Mean</td>
<td>0.038</td>
<td>0.046</td>
</tr>
<tr>
<td>Mean</td>
<td>0.037</td>
<td>0.044</td>
</tr>
<tr>
<td>Mean</td>
<td>0.068</td>
<td>0.065</td>
</tr>
</tbody>
</table>
TABLE 5.8.2 Comparison of the weight of ether solubles per unit leaf area on plants of *E. bicostata* grown at different temperatures and at different levels on the plants. (All differences of means given between temperatures or between leaf positions are significant at $P = .05$ or better.)

<table>
<thead>
<tr>
<th>Day Temp. at which plant grown</th>
<th>Replicate</th>
<th>Leaf Position on Plant</th>
<th>Percentage by which Weight of ether solubles of former exceeds that of the latter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants grown at 27°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper Leaves/Lower Leaves</td>
<td>A</td>
<td>0.038</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.038</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.038</td>
<td>22%</td>
</tr>
<tr>
<td>Plants grown at 21°C</td>
<td>A</td>
<td>0.036</td>
<td>43%</td>
</tr>
<tr>
<td>Upper Leaves/Lower Leaves</td>
<td>B</td>
<td>0.036</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.036</td>
<td>80%</td>
</tr>
<tr>
<td>Plants grown at 15°C</td>
<td>A</td>
<td>0.037</td>
<td>43%</td>
</tr>
<tr>
<td>and</td>
<td>B</td>
<td>0.037</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.037</td>
<td>80%</td>
</tr>
<tr>
<td>Plants grown at 15°C</td>
<td>A</td>
<td>0.037</td>
<td>43%</td>
</tr>
<tr>
<td>and</td>
<td>B</td>
<td>0.037</td>
<td>71%</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.037</td>
<td>80%</td>
</tr>
</tbody>
</table>
breadth of each leaf was measured with a millimeter scale, and each leaf was dipped momentarily, successively in each of the three beakers of ether. After each leaf of this group had been dipped, the contents of the three beakers were combined and placed in an evaporating flask and evaporated under reduced pressure to 25 cc. The contents of the large evaporating flask were then transferred to a 50 cc evaporating flask and taken to dryness. The weight of ether solubles taken from the 30 leaves was calculated. The area of each leaf was computed from the alignment chart (see Appendix 5/1 for preparation) from the length and breadth measurements. The total leaf area of the 30 leaves (both surfaces) was calculated and the weight of ether solubles per unit leaf area computed. This procedure was repeated for the leaves numbered 6-8 above the cotyledons for the same group of plants. The same method was followed to assess the ether solubles per square cm of leaf surface for each group of plants grown at each temperature.

The results of these measurements are presented in Table 5.8.1.

A statistical analysis of the means of Table 5.8.1, indicates the differences presented in Table 5.8.2, all of which are significant at $P = .05$ or better.
TABLE 5.83 Lesion number per unit leaf area on juvenile leaves of E. bicostata plants grown at different temperatures, and on leaves at different levels on the plants.

<table>
<thead>
<tr>
<th>Temperature at which plants grown</th>
<th>Leaf Position</th>
<th>Leaf Pairs 1-3</th>
<th>Leaf Pairs 6-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>27°C</td>
<td></td>
<td>0.348 ± 0.096</td>
<td>0.066 ± 0.019</td>
</tr>
<tr>
<td>21°C</td>
<td></td>
<td>0.295 ± 0.127</td>
<td>0.016 ± 0.007</td>
</tr>
<tr>
<td>15°C</td>
<td></td>
<td>0.118 ± 0.040</td>
<td></td>
</tr>
</tbody>
</table>
Comparison of weight of Ether solubles per unit leaf area and lesion number per unit leaf area on plants of *E. bicostata* grown at different temperatures and at different levels on these plants.

<table>
<thead>
<tr>
<th>Leaf position on the plant</th>
<th>Lower Leaves (1-3)</th>
<th>Upper Leaves (6-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature at which plants were grown (°C)</td>
<td>Mean weight of ether solubles per cm²</td>
<td>Lesion number per cm²</td>
</tr>
<tr>
<td>27°C</td>
<td>.038</td>
<td>.348</td>
</tr>
<tr>
<td>21°C</td>
<td>.036</td>
<td>.295</td>
</tr>
<tr>
<td>15°C</td>
<td>.065</td>
<td>.118</td>
</tr>
</tbody>
</table>
The figures for the weight of ether-solubles per unit leaf area are of the same order as those obtained for "wax" on a variety of plants (Martin, 1960).

The other 5 plants from each temperature treatment, not used in the assessment of ether solubles above, were spray inoculated with a spore suspension of P. eucalypti. They were incubated for 48 hours and assessed for lesion number per unit area at 40 days as previously described. The areas of these leaves were also computed by the use of an alignment chart described earlier in this experiment.

The results are presented in Table 5.8 3.

The two sets of data are set down for comparison in Table 5.8 4.

There is some degree of negative correlation between the weight of ether solubles per unit area and the lesion number per unit area. The significance of this correlation can be assessed by computing a correlation coefficient (Fisher, 1946). The value of $r$ (correlation coefficient) for this data is $-0.508$. For a significant correlation at $P=0.05$ with 5 pairs of observations $r$ must equal $0.8783$.

If in the above table the pair of observations for plants grown at $15^\circ$C are eliminated (the possible reason for doing this is suggested below) a correlation coefficient for the other 4 pairs of observations gives a value of $r = -0.913$. The
For 4 pairs of observations significance at the $P = .05$ level requires a value of $r = .9500$.

This analysis shows that the negative correlation between the weight per unit area of ether solubles and the lesion number per square cm on the juvenile leaves of *E. bicostata* grown at the three different temperatures is not significant at $P = .05$, if all the data are taken into the analysis. Nor is the correlation significant if only the data from the plants grown at $27^\circ C$ and $21^\circ C$ are considered. There were several reasons for eliminating the $15^\circ C$ plants in the second analysis.

The Electron Microscope Photographs of the carbon replicas of the leaf surfaces of plants grown at the temperatures indicate that the degree of glaucousness is probably less on those grown at $15^\circ C$, although the above data indicate that the weight of ether solubles from these leaves is very much greater (See Plates 5/3, 5/4, 5/5). The dried residues of the ether solubles of the $27^\circ C$ and the $21^\circ C$ are comparable in appearance; those from the $15^\circ C$ show, as well as the characteristic waxy smear, a great deal of brownish material. Finally the leaves from the $15^\circ C$ glasshouse showed a distinct reddish colouration along the midrib, major veins and leaf margins and even of the interveinal surfaces, no reddish colouration was present in the leaves of plants grown at the
Electron microscope photograph of abaxial surface of *E. bicostata* leaf on plant grown at 27°C phototemperature and 22°C nycotemperature. Note broad rods of wax.

Electron microscope photograph of abaxial surface of *E. bicostata* leaf on plant grown at 21°C phototemperature and 16°C nycotemperature. Note rods of wax somewhat finer than in Plate 5/3.
Electron microscope photograph of a baxial surface of *E. bicostata* leaf on plant grown at 15°C phototemperature and 10°C nycotemperature.
other temperatures. The leaves of the plants grown at 15°C might have been very different physiologically from the 27°C and 21°C leaves. The relationship between the weight of ether solubles and lesion number per unit area with the 15°C leaves might have been abnormal. A comparison of the two correlation coefficients indicates that this is likely. When all the data used in the analysis the negative correlation is only 57% of the value necessary for significance at the P = .05 level while if the 15°C treatment is ignored, then the correlation coefficient is 96% of that necessary for significance at the P = .05 level. (These two latter factors are)

The results of the earlier experiments showed the considerable effects of removal of glaucousness from juvenile E. bicostata leaves, on the lesion number per unit area caused by P. eucalypti on such leaves. The obvious implication is that glaucousness causes this effect, by a) its effect on spore germination and b) by its effect on wettability. Why has there been no statistically significant correlation between the weight of ether solubles per unit area and the number of lesions per unit area in this experiment?

There are many possible reasons some of which are discussed below. The further investigation of these would entailing considerable experimental work. Because of the
apparent significance of this glaucous layer in susceptibility such an investigation is very desirable.

An inspection of the Tables for the Correlation coefficient (Fisher, 1946) shows that a very high value of \( r \) (the correlation coefficient) is necessary where there are few pairs of samples in the data. A statistically significant correlation might have been obtained, had a greater number of samples been available for comparison.

In this experiment there was a basic assumption of a correlation between ether solubles and the factors affecting wettability and spore germination. (These two latter are factors which have so far been shown to be possible causes of reduction of number of lesions per unit area with presence of glaucousness). It is quite likely that this assumption is not correct. It is probable that the reduction of germination on glaucous leaves is the effect of a particular chemical or group of materials. The effect on glaucousness on wettability could be due a) the physical arrangement of the hydrophobic materials, b) the chemical nature of the hydrophobic materials themselves or c) a combination of both.

A comparison of the Electron Microscope photographs of the leaf surface carbon replicas indicates that the rods of "wax" are much longer and more numerous on the leaves of Burchill Nature, 180, 796-797, 1957.
plants grown at 21°C and 27°C, than those grown at 15°C. This physical difference could affect deposition. Variations in the physical structure of the waxes of Eucalyptus leaves has been described and discussed elsewhere (Hall, 1964; Chambers and Hallan, 1964). In the case of leaf "wax" of apple, the true waxes (acetone insolubles) and oils (acetone solubles), as percentages of the fresh weight of leaves, does not vary directly with variations in total leaf "wax" (i.e. the ether solubles) in samples of the same variety of apples at different times of the year. (Martin et al., 1957).

*Table 1 Composition of Apple Leaf Wax, 1957

<table>
<thead>
<tr>
<th>Variety</th>
<th>April 30th</th>
<th>May 27th</th>
<th>June 20th</th>
<th>July 15th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worcester</td>
<td>.73</td>
<td>.52</td>
<td>.43</td>
<td>.36</td>
</tr>
<tr>
<td>Pearmain</td>
<td>.51</td>
<td>.40</td>
<td>.37</td>
<td>.27</td>
</tr>
<tr>
<td>Orange Pippin</td>
<td>.50</td>
<td>.40</td>
<td>.35</td>
<td>.25</td>
</tr>
</tbody>
</table>

(Percent Fresh Weight of Leaves under the different temperatures and qualities and W° or W^+ represents the taxonomic families of cauliflowers, the Wax, ether "wax", acetone insolubles, acetone soluble, and oil (White Waxes, 19.11.08.09.07.08.09.06.07). W° Worcester Pearmain, W° Orange Pippin, W° hydrophobic materials (i.e. waxes and oils) at different temperatures.

It can be assumed that the true wax and oil are likely to be of major significance in the hydrophobic nature of the surface. The percentage which these constitute of the fresh weight of apple leaves, has changed independently of changes that have occurred in the percent which total "wax" has constituted of the fresh weight of the same leaves of the same species.

These observations suggest that in the experiment under discussion it would be necessary to:

1. Identify the material or materials in the leaf wax which is inhibitory to spore germination (Expt 16) and determine the variations in quantity of this substance per unit area under the different temperature treatments.

2. Determine to what extent the physical structure of the glaucous surface is significant in its hydrophobic qualities and observe changes in this with temperature.

It has been shown in the case of the "wax" of cauliflowers, that whether the "wax" occurs in the form of plates or rods is largely determined by the temperature of crystallisation (Whitecross, 1963).

3. Determine what proportion of the surface "wax" (i.e., ether solubles) is actually constituted by major hydrophobic materials (i.e., waxes and oils) at different temperatures. A correlation of the weight per unit leaf area of
hydrophobic materials and lesion number per unit area could more reasonably be expected, in view of the effect of these hydrophobic materials on spore deposition.

4. In the experiment and in points discussed under 2 and 3, there is the unestablished assumption that an increase in wax will result in the surface becoming more and more hydrophobic. It is more likely that at a certain level of physical orientation, or quantity of material, the surface becomes hydrophobic to a high degree and further increase in these factors has little effect on the wettability of the surface. In addition a distinction is necessary, between a smooth regular surface covered with a hydrophobic like layer and a leaf with its irregular surface topography. It was frequently observed on glaucous leaves that droplets of spore suspension accumulated along the margins of veins where they dried depositing their spores. If the leaf had been a smooth surface these droplets would have been shed completely.

It has been observed that the deposition of copper from Bordeaux mixture on leaves tends to reach a limiting value independent of the concentration in the spray. Electrokinetic interaction between positively changed particles and negatively changed leaf surfaces was advanced to explain this result (Rich, 1954). An
explanation along similar lines could be advanced to explain the possible non linearity of wax level and the degree to which the leaf surface is hydrophobic as indicated by spore deposition tests.

5. The discussion so far has also assumed the number of spores deposited is directly related to the degree to which the leaf surface is hydrophobic. The tendency of spore droplets to be shed by the leaf probably depends on the angle at which the leaf is oriented to the horizontal. In the case of a hydrophobic surface it is likely that this angle is very important in determining whether a spore droplet is dried in situ or shed. (This point was investigated experimentally later). Leaf angle as a factor in fungicide deposition has been discussed (Burchfield, 1959).

6. Finally, this discussion has taken account only of the axeny type factors affecting susceptibility and resistance to disease. Changes in the temperature at which plants are grown, obviously affect their morphology and the rate of physiological processes e.g. Photosynthesis (Bjorkmann and Holmgren, 1963). It is reasonable to assume that variations in temperature at which plants are grown may affect the physiological processes leading to active resistance e.g. phytoalexin production (Muller, 1958; Cruickshank, 1964). It could well be that in this
experiment the lesion number per unit area is more
dependent on the potential of plants grown at the differ­
ent temperatures to exhibit "active resistance", than on
changes in Axeny type resistance as a result of temperat­
ure of growth differences.

5. 9 Experiment 24 The significance of the angle at which
leaves are held in the deposition of spores of P. 
eucalypti from spore suspension on leaves of E. 
bicostata.

In the previous discussion it was suggested that the
angle at which a hydrophobic leaf surface was held to the
horizontal could considerably affect the number of spores
deposited on the surface. This suggestion was reasonably
easy to test, using juvenile glaucous leaves of E. bicostata.

4 Pairs of juvenile glaucous leaves of the same age
were taken from plants of E. bicostata, grown at 27°C phototemperarure and 22°C nycotemperature in the Ceres phytotron.
A piece of stiff cardboard 16" x 16" was taken and bent across
the centre. Pieces of 4" x 1" board were cut on a morticing
saw to give angles of 30°, 45° and 60°. These pieces could
be inserted in the bend of the cardboard to provide a surface
at 30°, 45° and 60° to the horizontal when the flap was attached
to the wood blocks with drawing pins. The leaves were attached
to the cardboard surface with paper clips.
<table>
<thead>
<tr>
<th>Angle at which leaf was held to the horizontal</th>
<th>Mean number of spores deposited per group of 25 L.P. microscope fields</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°</td>
<td>63.7 ± 9.86</td>
</tr>
<tr>
<td>30°</td>
<td>11.4 ± 2.47</td>
</tr>
<tr>
<td>45°</td>
<td>8.4 ± 1.23</td>
</tr>
<tr>
<td>60°</td>
<td>5.6 ± 0.37</td>
</tr>
</tbody>
</table>
The spore spraying nozzle, attached to a spore reservoir and a compressed air line, was clamped 18" above the center of the board described in the previous paragraph. The board was set at angles of 0°, 30°, 45° and 60° to the horizontal. At each setting two glaucous juvenile leaves were attached at marked positions on the board and sprayed for 2 mins with the spore suspension. The spray droplets were then dried on the leaves by placing the leaves, attached to the board, in an oven at 60°C for 10 mins. The leaves were removed from the board and the central portion from each leaf on either side of the midrib was cut out, cleared as described previously, stained and mounted in lacto-phenol. The spore deposition was assessed with a Low Power microscope. The leaf was worked over in a regular pattern, the number of spores per low power field on 4 groups of 25 fields per leaf piece were counted (i.e. 200 fields per leaf or 400 fields per angle of treatment). The number of spores in successive fields was often very variable. The spores, particularly on leaves held at an angle to the horizontal, frequently being clumped along vein margins.

Results are presented in Table 5.

The following differences of the means are significant at \( P = 0.05 \) or better: 0°/30° or 45° or 60°, 30°/60°, 45°/60°. The other differences are not significant at \( P = 0.05 \) level.
Discussion

This experiment emphasises the significance of the angle at which a hydrophobic leaf surface is held, on the number of spores of *P. eucalypti* which are successfully deposited on it. Elevating the leaf from a horizontal position to an angle of 30° reduces the number of spores deposited per Low Power Microscope field from over 2.5 to less than 0.5 per field.

On a seedling plant of *E. bicostata* with 12-14 pairs of expanded glaucous juvenile leaves it is common to find the following leaf orientation in relation to the horizontal.

- **Leaf pairs 1 - 2** - Leaf tips hanging down
- **Leaf centre and bases horizontal**

- **Leaf pairs 3 - 8** - Leaves as a whole horizontal

- **Leaf pairs 9 - 12** - Leaves oriented at an upward angle and above the horizontal varying from 30° to 60° in the upper leaf pair.

It is likely that leaf orientation will have a very significant effect on the number of spores deposited in the field and also in the previous experiments carried out in this Chapter. It seems reasonable to suggest that leaf orientation may be a very important factor in determination of the number of spores deposited on groups of upper leaves as against groups of lower leaves c.f. Expts 21 and 22.

Leaf angle to the horizontal as a consequence will probably also have a significant effect on the relative number of...
lesions per unit area, or the percentage area lesioned in upper by comparison with lower leaves, c.f. Expts 19 and 20. Leaf angle could also have been a variable in Expt 23. The plants which were grown at 15°C had only three pairs of lower leaves. These leaves had a noticeable horizontal orientation, they were relatively long and narrow by comparison with leaves of the same age from plants grown at 21°C and 27°C. They only turned down at the very tip, whereas leaves of similar position on plants grown at 21°C and 27°C had a downward angle throughout 50% of their length. This greater horizontality of the 15°C leaves, could have resulted in more spores than normal for their position on the plant being deposited on them. This could have resulted in a greater number of lesions per unit area than would have been expected for leaves with their concentration of ether solubles per unit area.

Under certain conditions leaf angle to the horizontal could be the most significant factor in relative disease susceptibility of particular plant varieties or of parts or organs of the same plant.

5.10 Leaf Age in relation to susceptibility

It has been evident in the results of some experiments
that the upper leaves on *E. bicostata* seedlings show significantly lower susceptibility to the disease caused by *P. eucalypti*, than do lower leaves on the same plant. In an experiment, not reported here in detail, it was also shown that in *E. grandis* seedlings there was a difference of approximately 200% (Sig at *P* < .05) between the number of lesions per unit area on lower leaves compared with the number on upper leaves of the same plants when the plants were artificially inoculated with *P. eucalypti* spore suspension.

In the case of *E. bicostata* seedlings it has been shown that there are very significantly larger numbers of spores deposited on lower by comparison with upper leaves. The relative degree to which the surfaces of the two leaf types are hydrophobic and the angle at which the leaves are held to the horizontal have been suggested as probable explanations for the differences in spore deposition. Per medium of differences in spore deposition the relative disease development could be affected. In the case of *E. grandis* experiment, the leaf angle to the horizontal varied very little between the lower and upper leaves. Also there was little apparent difference in degree of "waxiness" of the surface in the two positional groups of leaves in that species.

The factor of "age" difference between upper and lower leaves was mentioned previously, but no experiments with this
variable have been so far reported. In view of the result of Expt 23, where it was shown that there are significant differences in susceptibility to the disease caused by \textit{P. eucalypti} even between leaf pairs whose age differs by only one plastochron, this variable seems worthy of some attention. It seems unlikely that either angle to the horizontal or the degree of glaucousness could be so different between adjoining leaf pairs as to be the major factor determining degree of disease susceptibility.

Successive leaf pairs on a seedling of \textit{E. bicostata} differ in "age" in two ways. Each leaf pair is "physiologically older" by one plastochron than the leaf pair above it. Leaf pairs are produced when the apex is at different ages, i.e., at different stages in the ontogeny of the plant. In any consideration of the relationship of susceptibility to "age", these two types of "age" have to be distinguished.

The disease may be characteristic of a particular physiological age or of the organs produced at a particular stage of the shoots ontogeny.

The latent disease type infections may be considered as classical examples of disease associated with the physiological age of the organ. The pathogen only proliferates and the disease develops when a certain degree of physiological
Ripeness has been reached (Simmonds, 1941). The smuts of the grain crops are examples of diseases associated with a certain ontogenetic stage in the plant's life cycle. From its behaviour, peach leaf curl may also be a disease whose full expression depends on the leaf infected being from a particular stage in the ontogeny of the bud.

The ideal material for an experiment on the significance of physiological vis-à-vis ontogenetic age would be to produce, on the same plant, organs which were physiologically older, but ontogenetically younger than, adjoining organs. In the normal plant these two types of age parallel each other. It is possible by environmental modification to "physiologically age" a leaf, e.g., by light treatment. Any such treatment could have an effect on disease susceptibility independent of its ageing effect on the leaf, also such treatment could effect other adjoining leaves not subjected to the treatment.

In an attempt to overcome this inherent difficulty, two experimental approaches were made. The first involved taking a series of samples from a group of seedlings and assessing the relative disease susceptibility of leaves of the same ontogenetic age as the physiological age of the leaves increased (Expt 25). The second approach involved the preparation of identical twin cuttings and subjecting these to nutritional treatments which might simulate the effect of
physiological ageing at different rates in leaves of the same 
ontogenetic age.

5.11 Experiment 25 Significance of ontogenetic and physiol-
ogical age in the susceptibility of leaves of E. 
bicostata to the disease caused by P. eucalypti.

For this experiment 24 plants of E. bicostata were 
raised in the Ceres Phytotron glasshouses at a phototemperature 
of 27°C and nycotemperature of 22°C. At three weeks after 
pricking out the seedling into 5" pots, 6 plants with 3 pairs 
of fully expanded leaves were selected. This group was classed 
as being at an ontogenetic age of 3 weeks and the leaves were 
classed as a physiological age of 3 weeks. The seedlings were 
inoculated with a spore suspension of P. eucalypti, incubated 
and the disease allowed to develop for 40 days as previously 
described. At 40 days the lesion number per sqr cm of leaf 
surface was assessed.

At 5 weeks from pricking out a further group of 6 plants 
was selected. These plants had 6 pairs of fully expanded 
juvenile leaves. Of these 6 pairs of leaves 3 pairs had been 
formed when the plant was ontogenetically less than 3 weeks 
old (classed as 3 weeks of age ontogenetically) and 3 pairs 
had been formed when the plant was between 3 and 5 weeks of 
age ontogenetically (classed as 5 weeks of age ontogenetically). 
The three pairs of leaves of ontogenetic age 3 weeks, were now
TABLE 5. 11  Mean Lesion Number per unit Area caused by

*P. eucalypti on Leaves of *E. bicostata of
differing Physiological and Ontogenetic Age.

<table>
<thead>
<tr>
<th>Ontogenetic Age in weeks</th>
<th>Physiological Age in weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>.31 ± .086</td>
</tr>
<tr>
<td>5</td>
<td>.18 ± .042</td>
</tr>
<tr>
<td>9</td>
<td>.14 ± .027</td>
</tr>
<tr>
<td>15</td>
<td>.16 ± .012</td>
</tr>
</tbody>
</table>

Mean ± Standard Error

The table presents the mean lesion number per unit area caused by *P. eucalypti* on leaves of *E. bicostata* of differing physiological and ontogenetic age.
5 weeks old physiologically and the three leaves of ontogenetic age 5 weeks were classified as physiologically 3 weeks old. These plants were also inoculated, incubated and lesion number assessed per unit area at 40 days after inoculation. In the assessment the results were grouped into ontogenetic and physiological age groups.

This procedure of selection, leaf age classification ontogenetically and physiologically, inoculation, incubation and assessment was repeated at 9 weeks and 15 weeks when the plants were carrying 9 and 12 pairs of leaves respectively. The mean lesion number per sq cm and standard error of this mean was calculated for each ontogenetic and physiological age classification.

The results are presented in Table 5. 11 1.

The results are plotted in Figs 5. 11 1.

It is evident from these figures that the only significant differences in the means of lesion number unit area are between leaves of different physiological age. Leaves produced by the plant at different ontogenetic ages but inoculated at the same physiological age show only small non significant differences between the means of lesion number per sq cm.

Unfortunately as the plotted results show the variability in the data was very great. This could be the consequence of lack of genetic uniformity in the plant material. Also
The relationship between lesion number per sq cm caused by *P. eucalypti* and the age (physiological and ontogenetic) of *E. bicostata* seedling leaves.
ontogenetic age 3 wks.

ontogenetic age 5 wks.

ontogenetic age 9 wks.

ontogenetic age 15 wks.

Physiological age (wks.)

lesion no. per sq. cm.
different spore solutions had to be used for inoculation of the 4 successive groups of plants. It would be desirable to repeat this experiment using cuttings from a single plant to ensure genetic uniformity and to use single spore cultures for the inoculations. It would be necessary to raise the plants in a sterile environment so that lesions which appeared on the leaves could with confidence be attributed to inoculation with the pathogen. Using seedlings raised in the sterile phytotron had this advantage in this and other experiments performed.

From the results of this experiment it can be concluded that, within the range of physiological ages of leaves used, physiological age of leaf is a significant factor in the disease level produced by P. eucalypti on the juvenile leaves of E. bicostata. Conversely, within the range of ontogenetic ages used, there are no significant differences between the disease levels produced by P. eucalypti on juvenile leaves of E. bicostata produced when the bud is at different ontogenetic ages, of which have shown to occur on these leaf surfaces.

The results of this experiment can now be considered in relation to the results of experiments showing different degrees of disease susceptibility between leaves at different levels up the plant. The fact that leaves at different levels up the plant are of different physiological age could be a major
reason for the differences in susceptibility of such leaves. This does not explain why leaves of different age differ in their susceptibility.

Leaves of different physiological age could have different degrees of susceptibility for a variety of reasons. These can be summarised into two groups:

a. Axeny type resistance differences. It is possible that the degree of the Axeny type resistance due to the "Waxiness" of leaves changes with physiological age. In the field, abrasion of leaf surfaces would occur and this could be expected to reduce the effectiveness of the "wax" barrier. In the phytotron glasshouses, where the plants used in these experiments were mainly raised, there would probably be very little abrasion. Leaching of active antifungal materials or, if the materials are volatile, evaporation of them from the surface with leaf age, could be expected to occur.

These arguments could all apply equally well to either the inhibitor of germination or the hydrophobic materials, both of which have shown to occur on these leaf surfaces. The experiments on spore deposition on leaves of different levels is evidence that upper leaves are more hydrophobic than lower leaves. There is consequently a correlation between physiological age and the hydrophobic level of leaves. These two factors are both correlated with the degree of...
disease susceptibility of leaves at different levels on the plant. This would appear to be reasonable *prima facie* evidence that one of the effects of physiological ageing is to reduce the degree to which leaf surfaces are hydrophobic. As a consequence, more spores of the pathogen could be deposited per unit area on the leaf and consequently the level of disease incidence increased. These observations on reduced hydrophobicity with increasing age are in agreement with the results for the deposition of copper from Bordeaux mixture on the leaves of banana with increasing age. (Burchfield and *colla* Goenaga, 1957).

Further experiments indicating the degree of disease development in relation to spore load deposited should be sufficient to indicate that this is one of the methods by which physiological ageing affects susceptibility. If this work was combined with a more intensive attempt to correlate degree of disease development with the quantity of hydrophobic materials i.e. waxes and oils in the surface leaf wax, the then the mechanism of causes and effect would be clear.

b. Active Resistance. Leaves of different ages have different levels of physiological activity. It is evident from the studies on respiration and photosynthesis presented in Chapters 7 and 8, that as the leaf ages, physiologically, the photosynthetic rate gradually decreases and the respiration
rate slowly increases. These results are in agreement with the observations of other workers (Clark, 1961). It is reasonable to expect that changes in these basic metabolic processes will be paralleled by changes in the capacity of the leaf to perform other physiological activities, e.g., the production of phytoalexins affecting the degree of susceptibility of the leaf to the disease caused by the pathogen. In the case of peas, an investigation has shown an inverse relationship between pod maturity and pisatin production. The most mature pods used in this work were susceptible to *Monilinia fructicola* with the fungus sporulating on the infected tissue (Cruickshank and Perrin, 1963).

Physiological age of leaf is a significant factor in the degree of susceptibility of the leaf to the disease caused by *P. eucalypti*. It seems likely that one of the reasons for this change in susceptibility with age is that the degree to which the leaf surface is hydrophobic decreases with age. It is not possible, without further experiment, to eliminate the possibility that the general changes in physiology, which accompany leaf age, may also be important in determining the degree of susceptibility to disease caused by *P. eucalypti*. It is interesting to appreciate that the increased susceptibility to disease with age in the case of this pathogen is paralleled by the increased disease development with age.
which occurs in the "latent infection" group of leaf and fruit
diseases. In the latter case leaves are susceptible to
infection at various ages but the pathogen only develops in
the host as physiological ripeness is reached (Simmonds,
1941). In the case of the former susceptibility to infection
increases with physiological age and consequently degree of
disease development increases with age. Thus, with these
two distinct groups of pathogens, the end result is the same
although the path by which it is achieved is different. In
this regard it may be remembered that certain Septorias have
been recorded as causing latent infections of oranges, (i
with roots developed, were then placed in pots containing

5.12 Experiment 26 The effect of the mineral nutrient level
in which plants are grown on the susceptibility of
E. bicostata leaves to the disease caused by P. eucal-
ypti.

This experiment was commenced concurrently with Expt 25.
Its aim was once again to compare the significance of physio-

5

logical and ontogenetic age. The general technique used here
was to produce pairs of plants of the same genetic constitut-
ion carrying leaves of the same ontogenetic age and to subject
one member plant of each plant pair to a high and a low nutrient
level. It was thought that the low nutrient treatment might,
to some degree, simulate the effect of physiological ageing.
The raising of the seedling pairs was carried out by Mr Willing. The technique he used was briefly as follows. 10 seedlings of *E. bicostata* with 4 pairs of opposite small juvenile leaves were decapitated. This decapitation stimulated the production of lateral shoots from the axes of opposite leaf pairs. On each seedling the pair of axillary shoots from the topmost leaf pair was selected and allowed to grow to produce 3 pairs of opposite leaves, other shoots were pruned off as they developed. At this stage the paired shoots were marked, removed from the plant and used as cuttings. Roots developed successfully on 5 pairs of the shoots. These shoots, with roots developed, were then placed in pots containing washed sand. One of each shoot pair was watered with tap water twice per day, and a half strength Hoagland's solution once per week. The other member of each pair was watered with tap water once per day and half strength Hoagland's solution once per day.

A careful count was maintained of leaf production on the rooted cuttings so that it was possible to identify leaves of the same ontogenetic age on each cutting. At 2 months the two sets of cuttings had 5 pairs of fully expanded leaves of comparative ontogenetic age. All the cuttings were inoculated as described previously with a *P. eucalypti* spore solution. After 40 days the lesion number per square cm of leaf surface
TABLE 5.12 1 Degree of susceptibility of leaves of E. bicostata to the disease caused by P. eucalypti in relation to the nutrient level of the substrate on which the plants were grown.

<table>
<thead>
<tr>
<th>Plant No.</th>
<th>High Nutrient</th>
<th>Low Nutrient</th>
<th>Difference (H.N. - L.N.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1.030</td>
<td>0.472</td>
<td>+0.558</td>
</tr>
<tr>
<td>7</td>
<td>1.631</td>
<td>0.544</td>
<td>+1.087</td>
</tr>
<tr>
<td>8</td>
<td>0.857</td>
<td>0.591</td>
<td>+0.266</td>
</tr>
<tr>
<td>9</td>
<td>0.977</td>
<td>0.374</td>
<td>+0.603</td>
</tr>
<tr>
<td>10</td>
<td>0.589</td>
<td>0.271</td>
<td>+0.318</td>
</tr>
</tbody>
</table>
was assessed. The results for each group of 5 pairs of opposite leaves were combined. The results are presented in Table 5.123.1. The results of the treatments were subjected to a paired T test analysis. This test shows that the differences between the Lesion numbers per square cm on High and Low nutrient pairs is significant at $P<0.02$. The nutrient distribution between the leaves of plants grown on High and Low nutrient regime are more susceptible than leaves of comparable ontogenetic age on genetically identical plants grown under a Low nutrient regime. Further, leaves grown on a Low nutrient level will be the most susceptible. The relatively low number of lesions per unit area on plant No. 10 whether grown on Low or High nutrient regime suggests that this genotype may be more inherently resistant than the other genotypes used in the experiment.

The variation in the ratio of $H_N - L_N$, i.e., the difference column in the table to the corresponding Low Nutrient value (this varies from 2.0 in plants 7 and 9 to as low as 1.4 in plant No. 8), suggests a genotype/nutrient interaction. This conclusion is of value in itself. The assumption that the different nutrient treatments applied to the plant pairs would simulate the effects of physiological
ageing was probably unjustified. As leaves age some of their nutrient reserves are mobilised and transported to leaves in a more active metabolic state. (Biddulph, 1953). This mobilisation and transport would probably lead to an unequal distribution of nutrients in leaves of different ages. It seems likely that growing plants on unequal nutrient regimes would also produce leaves with unequal nutrient distribution between the treatments. It has not been shown however that this assumed nutrient difference is the critical factor in the susceptibility of leaves of different ages to the disease caused by *P. eucalypti*. In addition it is very doubtful if the physiology of leaves grown on a low nutrient level will be the same as the physiology of leaves grown on a higher nutrient but which lose their high nutrient status by transport until it falls to a low level.

The results of the experiment have clearly shown that nutrient status of plants is significant in the degree of susceptibility of their leaves to the disease caused by *P. eucalypti*. Since the comparison was between leaves of plants of identical genetic constitution and the same ontogenetic age this conclusion is of value in itself. The results are in general agreement with those for *S. apii* on celery where increased infection accompanied increased plant vigour (Thomas, 1921).
There is a considerable volume of rather contradictory literature on the effect of nutrition of the host on the susceptibility of a number of crop plants to particular diseases. (Stakman and Harrer, 1957; Stevens, 1960). In general with facultative parasites a reduction of disease level accompanies application of nitrogenous fertilisers, with obligate parasites high nutrient levels are usually accompanied by a high disease incidence. (Stevens, 1960).

The mechanism by which nutrition affects disease susceptibility in the case of P. eucalypti, is a matter of pure conjecture at present. The nutrition of the host could affect the significance of resistance to infection due to the glaucous barrier on the surface of leaves or it could affect the processes producing toxins characteristic of the active defence reaction mechanism of the phytoalexin type. This field is worthy of further investigation at some future time since it could contribute to the knowledge of the basic factors of host physiology making for resistance.

The indication of greater resistance in plant No. 10 than in other plants in the test was the first indication in any of the experiments so far conducted of higher resistance to the disease caused by P. eucalypti, associated with a particular genotype of E. bicostata. This matter is also worthy of further investigation.
The possibility of a host nutrient interaction suggested by the results would emphasise the importance of the host physiology in disease susceptibility. It emphasises the necessity for having large numbers of plants of uniform genetic constitution for use in disease susceptibility tests.

Further experiments not reported in detail. Expts 19 and 20 showed a significant difference between leaves at different levels on the plant in their susceptibility to the disease caused by _P. eucalypti_. Expt 25 showed an increasing susceptibility of leaves, of the same ontogenetic age, to the disease as the physiological age of these leaves increased. In discussion e.g. Expts 19 and 25, it has been suggested that factors other than those already investigated might be operative in determining disease susceptibility. It is possible that sterilisation destroyed material. An experiment was conducted to compare the suitability of leaves from different levels on the plant as substrates for the "in vitro" growth of _P. eucalypti_. 40 tubes, each containing 10 cc of water agar were taken. A cork borer was used to cut 1.5 cm diameter discs from Lower and Upper leaves of a _E. bicostata_ plant carrying 12 leaf pairs. The tubes were divided into 8 groups of 5. In each tube of four groups, a cork borer was responsible for the calculation of photosynthesis of 5 tubes, two discs of leaf tissue cut from lower leaves exchange measurements from which these calculations were placed. In each tube of the second four groups of 5.
tubes two discs of leaf tissue cut from upper leaves were placed. The tubes of agar after labelling were resterilised and when set were inoculated with uniform sized pieces of single spore culture of \textit{P. eucalypti}. The diameter growth of the organism was measured on the agar cultures at 2 weeks and at 4 weeks after inoculation. At 2 weeks, the mean diameter of the cultures on the upper leaves exceeded the mean diameter of those on the lower leaves by 12\% (not Sig. at \(P = .05\) level). At 4 weeks the mean diameter of the cultures on the upper leaves exceeded the mean diameter of those on the lower leaves by 5\% (not Sig at \(P = .05\) level).

These results indicate that there is no significant difference as a substrate in the leaves from the two levels used in this experiment, once the leaf material has been sterilised. It is possible that sterilisation destroyed materials which would have shown up such differences.

An experiment was also conducted to measure the photosynthesis of leaves of \textit{E. bicostata}, lightly wiped to remove waxiness, and of the same leaves before wiping. This work was carried out in conjunction with Mr. R. J. Cameron, a fellow Ph. D. student*. The Infra Red Gas Analyser equipment,

* Mr Cameron was responsible for the calculation of photosynthetic rates while I was responsible for the actual gas exchange measurements from which these calculations were made.
Effect of removal of glaucous wax upon rates of apparent photosynthesis in juvenile leaves of *E. bicostata*.
Light intensity

( x \times 10^4 \text{ ergs cm}^{-2} \text{ sec}^{-1} )
described and discussed in Chapter 6, was used for the measurements. The results of this experiment are illustrated in Fig 5.1.1.

At a light flux of $6 \times 10^4$ ergs cm$^{-2}$ s$^{-1}$, the wiped leaves have a photosynthetic rate 12½% higher (Sig at $P < .05$) than that of the same leaves before wiping. At a light flux of $12 \times 10^4$ ergs cm$^{-2}$ s$^{-1}$, the unwiped leaves have a photosynthetic rate 7½% higher (not Sig at $P = .05$) than that of the same leaves after wiping to remove the superficial glaucousness. The light intensities under which greater lesion number or greater lesioned area per unit area developed in wiped, by comparison with unwiped leaves of this species, Expts 18 and 19 ranged from 6 to $8 \times 10^4$ ergs cm$^{-2}$ hr$^{-1}$. These are light levels at which the wiped leaves have a higher photosynthetic efficiency.

The purpose of conducting this experiment was to indicate that changes occur in a basic physiological process following the light wiping of the leaves with cotton wool. A change in this process may in itself be important in the degree of disease development. It is more likely that increased photosynthesis in wiped leaves is indirect in its effect on degree of disease development. The amount of phytoalexin produced in a particular host-pathogen combination may vary with photosynthetic level in the host leaf. This
aspect of phytoalexin production does not appear to have been investigated. It was in the attachment of the experiments described so far in this chapter have dealt largely with the significance of Axeny type factors in infection of E. bicostata leaves by P. eucalypti and consequently the susceptibility of these leaves to the disease caused by the pathogen. It was decided to conduct a final experiment in which the significance of these factors was virtually eliminated. Such an experiment should show the extent to which the differential disease susceptibility of wiped and unwiped leaves and of leaves at different levels on the plant, depends on the reaction of the host to infection by the pathogen.

The aim of the experiment to eliminate the influence of the Axeny type factors giving resistance to infection was not satisfactorily achieved. Spores of P. eucalypti were germinated on 0.4 cm diameter discs of sterilised Whatman's No. 1 filter paper. The spores had been sprayed on the discs from a uniform spore suspension and counts of spores per unit area on the filter paper discs showed that a uniform spore load had been achieved. The spores were germinated for 48 hrs at 25°C in the dark in a humid environment. The intention was to attach the discs of filter paper with uniform loads of germinated spores to the lower sides
of wiped and unwiped leaves, and also to leaves at different levels on plants of E. bicostata. It was in the attachment of these discs that the experiment was unsuccessful. Two methods of attachment were attempted. In the first the discs were attached with pieces of cellophane. In the second they were attached by dipping in a weak solution of Gurr’s water mounting medium before placing on the undersides of the leaves. In both cases some of the discs did not attach firmly. One week after attachment the discs were removed. Unfortunately it was found that the degree of adhesiveness varied greatly. The attachment of the discs to wiped leaves was often so strong that the leaves were wounded in removing the discs. The same difficulty occurred with the soft upper leaves of E. bicostata plants. The number of unsuccessful inoculations or damaged leaves was so great that no comparison between leaves was possible in either experiment. It is necessary to develop a different technique for placing uniform loads of germinated spores on leaves before it will be possible to satisfactorily perform this experiment.

The requirements of such a technique are not easily fulfilled. If the germinated spores are placed on the leaves in discs of agar, the inoculum potential of the spore load might be such as to overcome any resistance due to the reactions of the host and pathogen. In addition it has been
shown that the media in which the spores of the pathogen are suspended affects the degree of pisatin production in the case of Pisum sativum (Cruickshank and Perrin, 1963). Further it is possible that changes in microclimate resulting from methods of attachment will affect the resistance reactions of host and pathogen. The ideal would be to apply the germinated spore suspension in uniform sized drops on the leaves and then allow these drops to dry. This would overcome the Axeny type factors affecting spore load and spore germination. The mechanical difficulty is in locating such drops on the undersides of hydrophobic leaf surfaces.

**Discussion**

The results of the individual experiments have been discussed. It will only be necessary to indicate the major conclusions which can be reached from them and indicate the significance of those conclusions in relation to the ecology and pathology of *P. eucalypti*.

The presence of a glaucous-layer on the surface of the juvenile leaves of *E. bicostata* is of considerable significance in the extent of the disease caused by *P. eucalypti* which occurs on such leaves.

It would appear that this glaucous layer is significant in the disease level achieved on the leaves because there is an inhibitor of spore germination present
in this "wax" material. The layer is hydrophobic and affects the number of spores deposited on the surface from a spore suspension. The removal of the "waxy" layer from the surface of E. tereticornis, a non glaucous species, also increases the deposition of spores on such surfaces from spore suspension. The degree of the effect of this removal is less in this instance than in the case of removal of the glaucous layer from E. bicostata. From these results it seems likely that the resistance of the leaves of the green juvenile species of eucalypts to the disease caused by P. eucalypti could be reduced by producing hybrids between these species and those with glaucous juveniles. It is to be expected that such hybrids, if selected for high degree of glaucousness, would show a higher level of resistance to the disease. The angle at which the juvenile glaucous surface is held to the horizontal is also a significant factor in the number of spores deposited on the hydrophobic surface. Varying degrees of hetero-blasticism are common in the eucalypts. The juvenile leaves of E. bicostata are short, sessile, opposite, held in a general horizontal position and with a pronounced bloom. The adult leaves are very long, lanceolate, petiolate, alternate, hanging and glossy. The mechanism by which the twisting of petioles results in
drooping or hanging leaves in the eucalypts has been described (Jacobs, 1955). The drooping of leaves, even if these are not glaucous, probably results in the shedding of considerable numbers of water dispersed spores. This feature should contribute to the disease escape of this type of foliage. Selection for a high angle to the horizontal of juvenile leaf, would also probably appreciably increase the resistance of certain species or hybrids to the disease.

There is a significant difference between the susceptibility of leaves at different levels on the plant to the disease. There is also a significant difference between the number of spores deposited on leaves at such levels from a spore suspension. This difference in susceptibility to disease of leaves at different levels is a function of physiological rather than ontogenetic age. The effect of physiological age could be due to its significance in the effectiveness of the hydrophobic layer or differences in active resistance may be associated with this physiological ageing. This observation has pathological importance because it means that it will be the older, less photosynthetically active leaves of seedlings which will be most heavily infected. In addition it has been shown that leaves of the same ontogenetic age on plants of identical genetic constitution,
but grown under different mineral nutritional regimes, show
different degrees of susceptibility. High nutrient plants
showing a greater susceptibility than low nutrient plants.
Once again the greater resistance of low nutrient plants
could be due to the effect of nutritional level on Axeny
factors affecting infection or on the host pathogen resistant
reactions. Higher disease incidence can be expected on
plants grown on high nutrient sites.

Sterilised upper and lower leaves of E. bicostata appear
to be of equal value as substrates for the "in vitro" growth
of P. eucalypti.

The investigations reported in this Chapter have
considered almost exclusively factors affecting resistance,
through their effects on infection regarded as a pre-penetr-
ation phenomenon. It is unlikely that those are the only
factors in eucalypt pathogen relationship which are signific-
ant in the resistance of various eucalypt species to P. eucal
ypti.

Further experimental work is necessary to investigate
the relative resistance of different species to the disease.
It is likely that some of the evident variability in resistance
at this level will be determined by the reactions of the host
to infection by the pathogen.
CHAPTER VI

The Physiology of Host Pathogen relations of Phaeoseptoria eucalypti on Eucalyptus

6.1 Introduction

A summary of the histology of the host pathogen relations has been given. Studies of host pathogen relations of diseased plants are fundamental to an understanding of symptom development and to an appreciation of the pathology of a particular host pathogen environment complex. A study of these relationships establishes an objective approach to both breeding and selection for disease resistance in plants as well as to the actions of systemic fungicides (White, 1957). A consideration of the histology of host pathogen relations, is a study of the end product of physiological changes. The histological study of reaction of certain barley varieties to powdery mildew illustrates this contention (White and Baker, 1954). The physiological changes which preceded and explained these histological reactions were investigated later. (Millerd and Scott, 1955; Scott, White and Millerd, 1957).

The combination of these two approaches, the histological
and physiological, has contributed greatly to an understanding of the resistance of varieties of barley to powdery mildew.

The increased interest in the physiology of diseased plants is readily understandable in view of the correlation observed between the respiratory pattern and disease resistance in grain crops (Millard and Scott, 1956; Samborski and Shaw, 1950; Walker and Stahmann, 1955). Whether the hypothesis of metabolic detoxification of the pathogen's toxins or metabolic production of materials toxic to the pathogen is accepted as the basic means by which a plant exhibits active resistance to a disease, energy is likely to be required to produce the necessary antitoxins or toxins, as respiratory increase is a general response of plant tissue attacked by pathogenic micro-organisms. The bulk of the investigations carried out have been with obligate parasites, but facultative parasites are also able to evoke this respiratory increase in host tissue. Chemical treatment and mechanical stimulation can also induce an increase in the respiratory rate of plants.
Evidence has also accumulated that during the period of respiratory increase the Hexose Monophosphate Pathway is predominantly operative (Daly, Bell and Krupka, 1961; Shaw and Samborski, 1957). The considerable reduction which occurs in the $C_6 : C_1$ ratio in infected by comparison with uninfected plants would indicate a replacement of the normal glycolytic pathway, by the hexose monophosphate pathway in the former. (Daly et al., 1961; Shaw and Samborski, 1957). It should be remembered however that both pathways are operative in normal plants, the glycolytic pathway predominating in the younger stages of growth and the hexose monophosphate pathway tending to replace it as the plants grow older and differentiate (Gibbs and Beevers, 1955).

Interest in the anabolic physiology of infected plants has been stimulated by observations on the accumulation of materials at infection courts (Shaw and Colotelo). This accumulation has been demonstrated most clearly with obligate parasites (Shaw, Brown, Rudd Jones, 1954). It has also been shown to occur with Septoria aesculi on Aesculus californica (Yarwood and Jacobson, 1955). Certain work has indicated that only uninvaded tissue in infected leaves showed enhanced starch accumulation (Wang, 1960). Early studies on photosynthesis during the infection
period showed a short but sensible increase in photosynthesis early in the infection cycle of plants by rusts (Montemartini, 1904; Grecusnikov, 1936). Allen (1942) in a study of the photosynthesis of wheat infected with powdery mildew, where a direct comparison was made of the rate of photosynthesis in infected by comparison with uninfected plants, showed a brief rise in photosynthesis in the first 4 days after inoculation with a subsequent fall to one third or one quarter of normal ones. By contrast, Sempio (1950) also working with powdery mildew on wheat has shown a rise in carbon fixation in the first 2-3 days of incubation, then a decrease followed by an increase again at the time of conidiophore and conidia differentiation and finally a drop to a very low value. Sempio (1959) when discussing these apparently contradictory results indicated that the contradiction is due to the manner in which the results of the two workers are expressed. This is partly true since Allen (1942) has given his results as absolute values, while Sempio (1950) has expressed his with the photosynthetic rate of the infected as a percentage of the rate of the uninfected. However examination of Sempio's data (1959) would indicate that the contradiction is a real one with the absolute values of CO$_2$ fixation at 8 and 11 days after inoculation being greater in infected than uninfected plants. No rise in respiration although infected green leaves
In a recent study (Livne, 1964) of the photosynthesis of healthy and rust affected bean and wheat plants, using a radioactive tracer technique for the measurement of $\text{C}^{14}$ fixation, it has been shown that, in bean leaves with a light infection, the rate of photosynthesis rose at 4-5 days after inoculation and then fell to $1/3 - 1/2$ of that of normal plants. Heavily infected bean leaves, or wheat leaves at any level of infection, did not show stimulation but exhibited an abrupt decline once sporulation was apparent. The most interesting feature of this investigation was the marked stimulation of photosynthesis in non infected leaves on infected bean plants. The degree of stimulation appeared to be related to the degree of infection on the infected leaves. No comparable results occurred with wheat but the uninfected cotyledons of safflower on plants with rust infected hypocotyls showed a 20% rise in photosynthesis. Sometime earlier it had been shown that rust infected hypocotyls of safflower elongated twice as rapidly as did uninfected hypocotyls (Daly and Sayre, 1959). Or other tissue. The use of cut pieces of an organ as did in a study which combined measurements on respiration and photosynthesis with assessment of activity levels of particular enzymes, (Scott and Smillie, 1963), etiolated barley leaves, infected with *Erysiphe graminis var. hordei*, showed no rise in respiration although infected green leaves...
of the same barley variety showed respiratory rises of 300-400 percent. This suggested a possible relationship between the respiratory rise and photosynthesis. At 144 hrs after inoculation photosynthesis in infected green barley leaves decreased to less than 50% of the uninfected level, while the respiration had increased 2½ times. The changes in enzyme activities indicated that the increased respiration in infected leaves was mediated through the hexose-monophosphate-pathway. This had been suggested by other investigators (Daly and Sayre, 1957). In etiolated barley leaves, although the growth of the fungus appeared normal, there was no rise in the respiratory level nor in the activities of enzymes associated with the hexose monophosphate pathway. The results suggest, that in this highly susceptible barley variety, the decrease in photosynthesis and respiratory increase are related.

Apart from investigations using radioactive tracer techniques, the measurements of gas exchange in the above investigations depended on the use of cut samples taken from leaves or other tissue. The use of cut pieces of an organ, as distinct from the intact organ, has definite disadvantages. It has been pointed out that, when other factors are non-limiting, rate of gas exchange from a leaf to the surrounding air is likely to be limited by the resistances in the pathway of the gas movement (Gaastra, 1959; Bierhuizen and Statyer, 1959).
1964). The use of a cut piece of leaf would greatly reduce the significance of these resistances in controlling the gas exchange rate. It can be expected that infection by a pathogen will alter the value of some of the resistances. Measurements of the absolute gas exchange rates of leaf pieces are not likely to be characteristic of the situation in vivo. Comparative measurements of infected and uninfected pieces are not necessarily indicative of the in vivo relationship, because the effectiveness of possible different resistances to gas-exchange in such leaves are not being taken into account.

In the first chapter of this series on physiology attention will be concentrated on the aim of the investigation, the materials used, the experimental design and some preliminary experiments. The second chapter will deal with the major experiments conducted on the time course of photosynthesis in infected and uninfected leaves and the third with the time course of respiration in infected and uninfected leaves.

6.2 Aims of the Experimental Work

1. The primary aim of the experiments was to study the time course of the respiration, photosynthesis and transpiration in leaves of eucalypts infected with Phaeoseptoria eucalypti and compare this with the time course of these
processes in uninfected leaves. Where possible the time course of changes in these physiological functions was related to histological observations on infected leaves. This aim could be summarised as the physiology of host pathogen relations of the particular pathogen on the particular hosts.

2. The secondary aim was to assess the overall significance of the results obtained for this pathogen host complex in host pathogen relations in general. A perusal of the literature on the physiology of diseased plants shows that many studies on respiratory changes in infected plant leaves have used obligate parasites either fungal or viral. Studies on changes in photosynthesis in infected plant leaves appear to have been conducted exclusively with obligate parasites. In such studies the pathogen is usually a virulent parasite which goes through its life cycle from inoculation to reproduction in less than two weeks. Studies of the time course of physiological processes in such host pathogen associations have the disability that the overall period of the host pathogen relationship is short. In the case of P. eucalypti the time course is 30 days. It could be expected that changes occurring in physiological processes of the host pathogen complex, would be better spaced out over
time from inoculation to pathogen reproduction. The possible relationship between changes in different physiological processes might be easier to distinguish in such a case than when the host-pathogen relationship is telescoped into a short period.

The significance of this second aim can be readily appreciated by reference to literature. The data of Sempio (1959), working with powdery mildew of wheat, is at intervals in a life cycle of 11 days. At 6 days his photosynthetic level is at an absolute minimum by comparison with other measurements on infected plants prior to the pathogen's reproduction. It is also at a relative minimum percent by comparison with the photosynthesis of uninfected plants prior to the pathogen's reproduction. His periods of maximum respiration correspond to his periods of maximum photosynthesis at 8 and 11 days.

Scott and Smillie (1963), working with powdery mildew on barley give results for 144 hrs only—6 days. Despite the difference in hosts, this time corresponds to the time of minimum photosynthesis and accelerated but not maximum respiration in Sempio's data. Is it possible that the relationship between photosynthesis and respiration which Scott and Smillie observed is the equivalent of Sempio's 6 day data? If it is, then the hypothesis they advance to
explain the results is barely tenable, because in Sempio's data a higher respiration level was associated with higher photosynthesis at a later stage in the infection, a fixed).

6. Materials and Instrumentation

I was fortunate in this investigation that most of the necessary apparatus had been collected and assembled by a fellow research student, Mr. R.G. Cameron. He has described in considerable detail in his thesis the apparatus and its calibration. Apart from a general outline of the instrumentation attention will be concentrated on modifications made to it for the purposes of this investigation.

The considerable difficulties involved in making accurate measurements of the gas exchange of individual leaves have been discussed (Gaastra, 1959). For the purpose of this investigation an instrument was needed which could quickly and accurately measure the carbon dioxide content of an airstream—the Infrared Gas Analyser (hereafter called an I.R.G.A.) is such an instrument. It was necessary to have a series of leaf chambers—in this case twelve chambers were used. In this investigation since it was intended to study variable in intensity from darkness through a series of

* Mr. Cameron had carried out a study on the effect of Light Intensity on Carbon Dioxide Assimilation of Eucalyptus leaves as part of a Ph.D. thesis he has submitted on "Light Intensity and the growth of Eucalyptus seedlings (with particular reference to Eucalyptus fastigata."
place the individual leaves, attached to the plants, in separate chambers and keep them there throughout the course of the experiment, the chambers had to be located in a fixed position in relation to one another and to the plants. In addition to the air lines for gas exchange measurements, since the leaf chambers were to be left closed, a reliable constant flow of fresh air had to be maintained over the leaf at all times when measurements were not in progress. For measuring purposes air had to be supplied to the leaf chambers at known controllable rates e.g. the rates for photosynthesis had to be much greater than for respiration if a reasonable chart deflection was to be made by the former. This air supply also had to be of fixed temperature, carbon dioxide content and humidity (Gaastra 1959). In the initial layout it was intended to measure transpiration. The humidity of ingoing airstream had to be such that after passing over the leaf, the air was not saturated. Apart from the impossibility of measuring the differential humidity, water would have condensed in the pipes leading from the leaf chamber to the analyser. A source of light, suitable in quality for photosynthesis, and variable in intensity from darkness through a series of intensities to a level above that necessary for light saturation of photosynthesis was desirable. The L.B. Growth Cabinet satisfies these requirements (Pescodetal, 1962).
It was also necessary to have an instrument which could measure the light intensities at different levels. Some instrumental method of recording the transpiration of the leaves enclosed in the chambers was necessary. The simultaneous continuous recording of the data for gas exchange, light intensity and transpiration necessitated an automatic recorder.

Diagram No. 6/1 gives a schematic outline of the necessary instrumentation outlined above. Portions of this instrumentation are given in more detail in Diags 6/2 6/3 and Plate 6/1.

An $S_{E}B_{K}$ type infrared gas analyser serial no. 6086 manufactured by the Infra Red Development Co., was used. The instrument is described in detail in the instruction manual (Infra Red Development Co. Ltd., 1960).

In this investigation the instrument was to be used for determining the difference between carbon dioxide in two air streams. For such a purpose the fine range of the instrument has a sensitivity of $\pm 0.0015\% CO_2$ full scale deflection if the centre of the circular chart built into the instrument is used as zero. In practice this sensitivity was considerably increased by moving the zero setting right or left of centre depending on whether respiration or photosynthesis was being measured. In practice the circular recorder chart was not used for recording. The transmitter
Diagram showing schematic layout of instrumentation for measuring photosynthesis, respiration and transpiration of individual leaves. (see detail in following diagram.)
Air Supply

Temperature & Humidity Stabilisation

Air Mixing Flask

Splitting Airstream

Vertical Manometers. Flow Rate.

Psychrometer

Blank Chamber

Leaf Chamber

Psychrometer

Horizontal Manometers

Removal of Moisture

I. R. G. A. Comparison of Airstreams

Bleed off Air in excess of 15 L. per hr
Schematic diagram of instruments used for measurement of photosynthesis and respiration.

Key to components:

F. Filter flasks
P. Air pump
$V_1, V_2, V_3, V_4$ Three way stop cocks
$WB_1$ and $WB_2$ Water baths to set temperature and humidity of incoming airstream
M.F. Air mixing flasks
O.V. Overflow valve
V.M. Vertical flow meters
B. Blank chamber
L.C. Leaf Chamber
H.M. Horizontal flow meters
Leaf chamber assembly layout (see detail in diagram 6/5).
Showing portion of air circulation system. The I.R. G.A. unit is right of centre. Note the water removal unit and the air flushing unit of the I.R.G.A. in the centre and top centre of the photo.
in the servo system provides a 0-2.5 mV signal output, this system was connected to a potentiometric recorder set up on a table at some distance from the instrument.

The optical compartment and the absorption tubes not in use (in this case the short coarse range tubes of the analyser) have to be constantly flushed with clean, dry, carbon dioxide free air. If dust, water or CO₂ particles, other than those fed into the absorption tubes in use, get into the path of the infra red ray between the source and the analyser head, these will result in readings not due to the CO₂ particles in the absorption tubes in use. A larger circuit for removal of water and carbon dioxide from this flushing stream, than that recommended by the manufacturers, had been installed. An additional modification introduced here was to dry the anhydrous Calcium chloride in an oven at 125°C for at least 5 days before use in the circuit. Although the calcium chloride has a higher water content at equilibrium than the other driers, ("Drierite", self indicating Calcium sulphate and anhydrous magnesium perchlorate) (Hodgman et al, 1962) which followed it in the circuit, because of its bulk it was responsible for the removal of most of the moisture from the circuit. If the Calcium chloride was dried as suggested and the flushing circuit was free of leaks, then the drying materials would last for
at least 2 weeks without any necessity for changing the contents. The final tube in this circuit contained Carbo-sorb (self indicating soda lime) for removal of CO₂ in a second. The I.R.G.A. had been adjusted in accordance with manufacturers directions before this investigation commenced. Apart from a failure in the servo system on one occasion, no major adjustments were made. This procedure ensured that the air reaching the leaf chamber had to be at a constant humidity and temperature otherwise these factors could affect transpiration (Bosian, 1963). The air from the pump after filtering was bubbled through a series of 4 bottles containing distilled water. These bottles were set in a constant temperature water bath with a refrigeration coil inside. The temperature control of the water bath was hour adjusted so that, with the refrigeration unit operating its...
constantly, the temperature of the water in the bubbling bottles was 8°C. From this first bath the air emerged saturated at 8°C. It was then led through a copper coil in a second water bath. The temperature of the second bath was varied when necessary so that the air on emerging had a temperature of 27°C as measured on an alcohol thermometer inserted in the air line. This procedure ensured that the air reaching the leaf chamber was at 27°C (the temperature set on the growth cabinet control) and that it was of a constant relative humidity of approx. 30%.

Flow rates through the leaf chamber were varied according to whether photosynthesis or respiration was being measured. This variation was achieved by using the calibrated vertical flow metres with a series of jets of varying orifice diameter.

For photosynthesis, in the case of *E. bicostata*, flow rates of 120-150 litres per hour were used. The higher rates being necessary for larger leaves. With the larger leaves of *E. tereticornis*, flow rates of up to 300 litres per hour had to be used in the initial stages of measurement, when photosynthetic rates were high; otherwise the deflection due to CO₂ added to the airstream would have put the recorder off chart. For respiration flow rates of 35-50 litres per hour were used. Higher flow rates for respiration measurements...
would have meant smaller chart deflections which would have been difficult to record accurately.

The wet and dry bulb thermocouple psychrometers used for transpiration measurements are only accurate for recording wet bulb depression if the flow rate over the wet bulb exceeds 2 metres per sec. The psychrometer dimensions were designed to achieve this rate of lateral flow, with a volume flow of 100 litres per hour. Thus light transpiration and photosynthesis measurements were recorded simultaneously, measurements of respiration and dark transpiration had to be carried out separately.

The I.R.G.A. in this investigation was used in an open circuit system, in contrast to the closed circuit system preferred by some workers, (Clark, 1961). An objection advanced against the use of an open circuit is that relatively high flow rates are necessary in an open circuit (Keller and Samish, 1964). According to the objection this means putting large quantities of air through the analyser. Since at above 120 litres per hour some compression of gases within the analysis tubes occurs, the open system is less accurate than a closed system. This difficulty was overcome by using only a small sample of each of two air streams for comparative analysis in the I.R.G.A.. This sample was controlled and balanced at 15 litres per hour by bleeding off the excess air above
The rate of flow through the leaf chamber and the blank airstream was set by the vertical flow metres (200 litre per hour jets were used for photosynthesis and 50 litre per hour jets for respiration) and the flow of both streams to the analyser controlled by the horizontal flow metres at 15 litres per hour. The calibration of the two sets of flow metres had been carried out previously for a variety of jet sizes (Cameron, 1964).

The major modification made in the air circuit, apart from those for accurately adjusting the humidity, was the introduction of the psychometers for measuring humidity. The design of a simple wet and dry bulb thermocouple psychometers has been recently described and their use in the calculation of transpiration rates outlined (Bierhuizen and Slatyer, 1964). Instruments for the measurement of transpiration of single leaves are not common and the immediate choice appeared to be between these psychometers and Lithium Chloride elements. The output of the former is in milivolts which could be recorded on the available potentiometric recorder. The output of the latter, in ohms and for a continuous record a resistance recorder would have been necessary. The psychometers, a slightly modified version of the above design, were made in the workshop of Physics Dept. of the
Detail of design of the wet and dry bulb thermocouple assembly.
A. N. U. School of General Studies. The four thermocouples - copper/constantin - were made in the workshop of the Ceres phytotron. A sketch diagram of the psychometer is given in Diag No. 6/4. The thermocouples had a cold junction and very long leads from the growth cabinet to the recorder. The thermocouples were tested for accuracy and comparability by inserting them in distilled water, with their cold junctions in tubes immersed in a vacuum flask containing iced distilled water, and reading the temperatures as recorded in millivolts at 2° intervals over a range from 15°C to 35°C. The millivolt readings were compared with the water temperature as read with a fine scale mercury thermometer. One thermocouple which was obviously inaccurate was replaced and retested. The four thermocouples were comparable in their readings for the different temperatures. Their readings for the particular temperatures showed a maximum difference of +0.5°C from the temperature as determined by the thermometer. When the psychometers were in use they were inserted in the air circulation circuit as shown in Diag No. 6/1. The psychometer for determining the humidity of the air before passing over the leaf was placed on the "blank" airline while the psychometer for measuring the humidity of the air after passing over the leaf was placed on the outpipe of the leaf chamber line. This setup ensured that the humidity of the ingoing modified
The airstream was not raised before reaching the leaf chamber by passing over the wick of the wet bulb thermocouple. The thermocouples were shielded with aluminium-foil from the light to eliminate variations in millivolt output due to absorption of light energy. At this level the variations in light intensity from a wet bulb and dry bulb thermocouple were very small. With an I.R.G.A. the content of CO₂ in an airstream can only be accurately determined if water vapour is removed.

Water vapour molecules have an absorption of infra red radiation in the wavelength used in the analyser. The water vapour was removed from the airstream before the stream was led into the analyser by output of the thermopile was in millivolts led Condensation tubes whose bases were in contact with a calimixture of crushed ice in alcohol in vacuum flasks (Camthis mixture maintained a temperature of -24° to -20°C).

2. This was followed by U-tubes packed with "Dricite" the desiccating Calcium sulphate-Equilibrium at 0.005 of featmg H₂O/l. (Hodgman et al., 1962). Gasstra (1959), No 3. 3. Finally the air stream passed through U-tubes packed with anhydrous magnesium perchlorate. (This has an addition weighable amount of water at equilibrium) Hodgman et al., 1962). As shown in Diag 6/5 the leaf chambers

The uniform environment for the leaf chambers, psychometers etc. was provided by placing these in an L, B, growth cabinet (Pescod et al., 1962). The cabinet had been modified
(Cameron, 1964), so that matching pairs of the 28 T L-33 fluorescent tubes could be switched off, this gave satisfactory control of light intensity. In the cabinet the leaf chambers in their carrier were set at approximately 24" below the light box. At this level the variation in light intensity from the front to the back of the cabinet was a minimum (Pescod et al., 1962).

The light intensity inside the cabinet was measured with a Kipps G-19 constantan-manganin thermopile. The thermopile was supported in the cabinet at the level of the leaf chambers. The output of the thermopile was in millivolts and could be recorded on the potentiometric recorder. The calibration of the thermopile had been carried out previously (Cameron, 1964).

The leaf chambers (12 in all) were constructed to the design shown in Diag 6/5*. The design incorporates most of the features of the chambers recommended by Gaastra (1959). No water jackets were incorporated in the chambers since check measurements showed that, at the flow rates used, air temperatures in the chambers were within 1.2°C of those of the growth cabinet. As shown in Diag 6/5 the leaf chambers

* These chambers were made by the workshop of the Physics Department of the School of General Studies A.N.U.
Leaf Chamber assembly detail. Diagram shows the lower half of the two identical pieces which make up a single leaf chamber.
consisted of two identical halves which could be clamped together with screws and butterfly nuts with an O-ring seal set in a continuous groove around the joint between the two halves. The leaf was fixed above and below in the centre of the chamber on nylon fishing line threaded through holes in the baffle plates. The small opening at the end of the chamber, through which the petiole of the leaf entered the chamber, was sealed with modelling clay and the halves then clamped together with the butterfly thumb screws.

The air circulation through the chamber was designed to give good turbulence and consequent mixing. The holes bored along the copper inlet and outlet tubes faced the outside wall of the chamber. The air entering the chamber was reflected from the outside wall of the chamber, through the baffle plates in which the circulation holes were bored at an angle of 45°. The stream passed over the leaf, through the holes in the other baffle plate (at 45°) against the outside wall of the leaf chamber and was reflected back into the holes in the outlet copper pipes.

A general air circulation, as well as a measuring line circulation, was attached to each leaf chamber. Each line was connected by a three way petrol tap to the inlet and outlet tubes of each chamber. On the general circulation line the air was brought to the tap in plastic tubing,
Initially from a small electric air pump, but later from the compressed air supply in the Botany Department. The general circulation air, after passing through the leaf chamber was released into the growth cabinet. The measuring inlet air line was in copper tubing from the vertical manometers, setting the flow rate, and after passing over the leaf was taken back to the horizontal manometers in copper tubing.

The 12 leaf chambers were used in two parallel series of six. The chambers were arranged in opposite pairs so that an uninfected leaf from a plant could be placed in one chamber and the opposite, infected, twin leaf in another chamber (see Diagram 6/3). An angle iron frame was built up to carry the two banks of 6 chambers, so that the 12 chambers together with their connecting tubing etc. could be mounted as a unit.

This frame allowed a degree of movement necessary to bring the leaf chambers into position against the plant stem with the petiole through the opening which was then sealed. When setting up, the chambers were individually divided into their two parts and separated from their taps at the screw joint. The lower part of the chamber was placed in position under the leaf and a quantity of modelling clay, sufficient to seal the hole, placed around the petiole. The top was then placed on the leaf chamber and the inlet and outlet pipes connected to their taps. The lid of the chamber was then screwed down
tight, ensuring that no strain was placed on the leaf petiole. Once sealed in the chamber the leaf was left in position for the duration of the experiment i.e. 40 + days.

The output of the I.R.G.A., the thermocouple psychometers and the Kipp thermopiles was all in a millivolt range of 0-2.5 mV. A Brown Electronik 153 x 89 potentiometric recorder (0-2.5 mV) was used to continuously record the output from these instruments. The recorder was adjusted for the operation of 8 of the potential 24 channels, although only 6 of those channels were actually used for instrument measurements. The chart speed on the recorder was 6 ins per hour.

The plants for use in these experiments were raised in the Ceres phytotron glasshouse in perlite (phototemperature 27°C, nycotemperature 22°C, 16 hour photoperiod, 8 hours + photosynthesis, 8 hours darkness and receiving half strength Hoagland's nutrient solution in the morning and demineralised water in the evening). During the period of the experiment, apart from the times when measurements were being made and conditions sometimes varied, (e.g. darkness during respiration) the plants were kept at a phototemperature of 27°C, nycotemperature 22°C, 8 hours photosynthesis, 16 hours photoperiod and 8 hours darkness. The watering regime was the same as in the phytotron.
6.3.1 Sequence of operations

When measurements were to be carried out, the plants were given 2 hours to adjust to the light environment (darkness for respiration, full light intensity for photosynthesis) before commencing recordings. During this period the freezing mixture for removal of water was renewed. The leaf chamber and the blank chamber were bypassed and the zero shutter of the I.R.G.A. was adjusted to give a maximum use of the chart space for deflection either positive or negative according to whether CO₂ production or consumption was to be measured. This period ensured that a stable zero was obtained on a comparison of the two air lines. After this settling down period the air streams were switched to a leaf chamber and the blank and the I.R.G.A. allowed to settle again until a stable reading for CO₂ differential was obtained for a 10 minute period. The measuring stream was then switched to the next leaf chamber, and the process repeated. After the CO₂ differential had been measured for half the chambers the zero setting of the I.R.G.A. was checked, since the movement of the zero with small changes in the CO₂ content of the incoming air stream is the most likely source of error.

6.3.2 Calibration of Instruments

The calibration of the I.R.G.A. output, in divisions (1/10 inch) on the potentiometric recorder had been carried
out previously (Cameron, 1964). For this purpose a gas mixture supplied by the manufacturers containing 0.0012% CO₂ was passed through one side of the machine and CO₂ free air through the other. The differential between the two airstreams was recorded on the Brown Electronik Recorder. The differential measured in parts per million CO₂ was

\[ D \times 0.275 \]

where \( D \) = units of deflection on the recorder.

Calibration of the Kipps thermopile.

The calibration of the potentiometer recorder for light output in the 400-700 nm wave band had been carried out (Cameron, 1964). The incandescent lights were turned off while carrying out measurements because these could not be turned down in relation to reduction of intensity from the fluorescent tubes. (If these had been left on at the lower fluorescent light intensities the spectral characteristics of the light in the cabinet would change). The thermopile output on the recorder was calibrated against an EEL light metre with the metre and the thermopile mounted side by side in the growth cabinet. The calibration was

One recorder unit = \( 1.56 \times 10^{-3} \) ergs cm\(^{-2}\) sec\(^{-1}\)

6.3 Accuracy of measurement and reproducibility of Results

These had been tested previously (Cameron, 1964) and no
further specific tests were carried out. On the point of reproducibility however the experiments themselves are sufficient evidence. Measurements were usually taken at 2 day intervals and Figs 7.2 and 8.3 indicate there were no significant differences between the means of measurements of photosynthesis or respiration over considerable periods.

In an investigation, (Cameron, 1964) it had been shown that with individual plants, there was a initial high level of photosynthesis for 1½ - 2 hours after the lights came on. The rate then settled down to a more or less stable level. Measurements of photosynthesis or respiration were never commenced at less than 2 hours from the start of the particular light regime.

During the experiments evidence accumulated that the rate of respiration showed a definite tendency to reduce during the dark period. At the end of a 6 hour period the respiration in some plants was only 60% of its value in the same plants at the beginning of the period. This considerable reduction was possibly due to the reduced substrate level of the leaf. In this particular investigation it was not necessary to attempt a solution to this difficulty. The interest in the investigation was the comparison between uninfected and infected leaves on the same plants. The measurements on the pair of leaves on each plant was completed before passing
onto the next plant consequently the time interval separating the commencement and conclusion of measurements which were to be compared was never more than 45 minutes. There was no measurable change in respiration in this time interval.

A further factor which affected absolute respiration rates, but which was not of importance in this investigation, was the period of light which preceded the dark treatment. A period of 8-12 hours light preceding the dark period gave a much higher respiration rate than 1-2 hours light followed by darkness, even if the plants were allowed 2 hours in each case to settle down in the dark before measurements were commenced. It has been shown elsewhere that the period of photosynthesis preceding is significant in the dark respiration of spruce and beech (Pisek and Tranquillini, 1954). Since photosynthesis and respiration were usually measured on alternate days throughout the experimental period, the pre-dark treatment received was conditioned by this measurement procedure.

This investigation was concerned with the comparison between infected and uninfected leaves, not the absolute values of respiration, and since all leaves received the same treatment, these variations were of no immediate significance. In an investigation where absolute values were critical, a pre-dark light intensity experiment to
determine the optimum light treatment for maximum dark respiration would have to be carried out. Even in an experiment of the present type, if the regime of light and dark were not conditioned as was the case here by the types of measurements being made, such an experiment would be worthwhile.

It is possible that the differences between the respiration of infected and uninfected leaves would be magnified if the pre-dark, light treatment was such as to have each leaf respiring at its maximum rate.

6. 40 Preliminary Experiments

Experiment 27 Photosynthesis in relation to Light Intensity in E. bicostata and E. tereticornis

The light intensity output of the T.L.-33 tubes decreases with age (Pescod et al., 1962), consequently it was desirable to determine the light intensity for maximum photosynthesis in the species to be used in the comparative experiments. These comparative experiments were to be conducted over a 40 + day period and it was desirable that the light intensity in the growth cabinet throughout that period should be adequate for maximum photosynthesis.

For this purpose 2 plants each of E. tereticornis and E. bicostata were taken. The appropriate plant was placed with one leaf of each pair in opposite leaf chambers and allowed to settle down in the full light intensity environment...
for 2 hours. During this period a stable zero was achieved on the analyser. The blank and first leaf chamber were then cut into the circuit and the deflection, due to CO₂ removal from the air stream at this light intensity, allowed to settle to a stable value. The deflection was then read off, 2 of the fluorescent tubes turned off and the deflection allowed to settle again to stable value, this process was repeated for the particular leaf until turning off pairs of lights gave a significantly measurable reduction in deflection. Further reduction of light intensity was usually continued for several more pairs of tubes, showing further reduction of CO₂ removal from the air stream with reduced light intensity. The deflection values were then converted to the rate of apparent photosynthesis by the following formula.

\[
\text{Rate of apparent photosynthesis} = \frac{D \times 0.275 \times \text{Flow Rate}}{\text{Leaf Area}}
\]

The rate from this formula is in \( \text{mm}^3 \text{CO}_2 \text{ cm}^{-2} \text{ hr}^{-1} \) where flow rate is in litres per hour, the leaf area is in \( \text{cm}^2 \) (a deflection of 36.6 units on the recorder) and \( \text{D} \times 0.275 \) is the deflection in parts per million CO₂. The absolute rates of apparent photosynthesis at different light intensities were then expressed as a percentage of the maximum of apparent photosynthesis of the particular leaf and these values were made of the rates of apparent photosynthesis in infected and uninfected leaves, the light level did not fall below 75
plotted against light intensity in \( \text{ergs cm}^{-2} \text{ sec}^{-2} \) calculated from the deflection values, recorded by the Kipps thermopile.

The value for respiration for each plant was determined in the dark at a flow rate of 35 litres per hour. These measurements were repeated for the two pairs of leaves on each plant of both *E. tereticornis* and *E. bicostata*. From this data curves were produced which showed the rate of apparent photosynthesis in relation to light intensity for each of the species. From these curves the level of light intensity at which apparent photosynthesis reached a maximum could be read off. In the experimental period so long as the light intensity recorded did not fall below this value it was assumed that changes in photosynthesis were not due to reduction in light intensity with ageing of the T.I-33 tubes. These photosynthesis/light intensity curves are shown in Figs 6.401 and 6.402.

For *E. tereticornis* light intensities of \( 8.8 \times 10^4 \text{ ergs cm}^{-2} \text{ sec}^{-1} \) (a deflection of 56.4 units on the recorder) and above were suitable for maximum photosynthesis. For *E. bicostata* light intensities of \( 11.1 \times 10^4 \text{ ergs cm}^{-2} \text{ sec}^{-1} \) (a deflection of 71.1 units on the recorder) and above were suitable for maximum photosynthesis.

In the experiments following, where a comparison is made of the rates of apparent photosynthesis in infected and uninfected leaves, the light level did not fall below 75
Variations in rate of apparent photosynthesis of E. tereticornis leaves with light intensity.
apparent photosynthesis as percentage of maximum rate

light intensity below which apparent photosynthesis is less than maximum
FIGURE 6.402

Variations in rate of apparent photosynthesis of *E. bicostata* leaves with light intensity.
Apparent photosynthesis as percentage of maximum rate

Light intensity below which apparent photosynthesis is less than maximum

Light intensity (x10^4 ergs cm^-2 sec^-1)
Units, on the potentiometric recorder, i.e. $11.7 \times 10^4$ ergs cm$^{-2}$ sec$^{-1}$.

Two other factors, which could affect saturating light intensity for photosynthesis, were not tested although they could possibly have had some significance in the investigation. The first of these is the change in the saturating light intensity, with increasing physiological age. In general photosynthesis per unit leaf surface, increases with physiological age of leaves of several deciduous tree species up to full leaf expansion, then decreases as the leaves age. (Kramer & Kozlowski, 1960; Richardson, 1957). In one species of oak photosynthesis lagged behind leaf expansion (Richardson, 1957). In conifers the rate of apparent photosynthesis in new foliage of White Spruce and Balsam Fir at a light intensity of 1000 - 40 ft-candles, increased from a negative value early in the growing season, to a maximum in August (Clark, 1961). In the case of a number of conifers it has been shown that photosynthesis per 100 needles decreases with needle age after the end of the first year (Freeland, 1952).

The other possible variable is a change in saturating light intensity with the progress of infection i.e. an infected leaf might achieve its maximum photosynthesis at a higher or lower light intensity than a comparable uninfected leaf. This possibility does not appear to have been investigated by other
of the for the purpose of this investigation it was considered
light intensities between $8.8 \times 10^{-4}$ ergs cm$^{-2}$ sec$^{-1}$
for E. tereticornis and $11.1 \times 10^{-4}$ ergs cm$^{-2}$ sec$^{-1}$
for E. bicostata were saturating for apparent photosynthesis
irrespective of changes in the physiological age of the leaves
during infection and of changes induced in the infected leaf.

6.42 Experiment 28: Respiration rate of Phaeoseptoria
eucalypti.

In the experiment envisaged on the effect of infection
by Phaeoseptoria eucalypti on the respiration of eucalypt
leaves, the respiration measured would be the respiration of
the host-pathogen complex. It was to be expected from the
results of other workers that the infected leaves would show
an increased respiration by comparison with the uninfected.
(Uritani, Akazawa, 1959). The experiments of workers using
the powdery mildews, whose superficial mycelia can be readily
separated from the host before measurement, have shown that
in this case the enhancement of respiratory rate in infected
plants can be attributed in part to the rise in host tissue

In host-pathogen associations where the two members of
the complex are not so readily separable mechanically, some
other means must be employed to assess the respiration of rate
the pathogen. Some workers have used an artificial culture of the organism for this purpose (Weir, 1962). In a preliminary experiment on respiration comparing infected and uninfected leaves at 30-35 days from inoculation it had been established that the respiration of the infected leaves was higher at this age, (Chapter 8). It was decided to assess the respiration of the fungus at this age, after artificial inoculation, on killed leaves.

Leaves were selected from the 3rd node and from the 8th node of 3 separate E. bicostata seedlings with 12 pairs of fully expanded leaves. The outline of the leaves was traced and their area determined. Each leaf was marked and placed in a separate petri dish, under a bell jar with a small quantity of propylene oxide in a separate dish. The leaves were exposed to the propylene oxide fumes for one hour. The dishes containing the leaves were then transferred to a sterile room where they were left exposed to the air for 48 hours to allow the propylene oxide fumes to clear. The leaves were then transferred to petri dishes containing a layer of vermiculite covered by a sheet of Whatmann's filter paper which had been soaked with sterile distilled water. The leaves were inoculated with a heavy spore suspension of P. eucalypti. The leaves were incubated in the dark at 25°C for 29-30 days. Each leaf was then placed in a separate
TABLE 6.  Respiration rate of *P. eucalypti* at 29 - 30 days after inoculation on leaves of *E. bicos-tata* killed by propylene oxide before inoculation.

<table>
<thead>
<tr>
<th>Leaf Position</th>
<th>Leaves from 3rd node</th>
<th>Leaves from 8th node</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean respiration rate in cubic millimetres of 10 per cm of leaf surface per hour</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.710 ± 0.181</td>
<td>0.792 ± 0.124</td>
</tr>
</tbody>
</table>

*Each result represents the mean of 3 leaves from the same nodal position.*
leaf chamber which was sealed. The respiration of the fungal infected leaves was determined in the dark. After the respiration measurement the leaves were examined. The pathogen was fruiting prolifically on the tissue and random sections of the leaves showed that they were thoroughly permeated by fungal hyphae. The rates of respiration were calculated in \( \text{mm}^3 \text{cm}^{-2} \text{hr}^{-1} \). The rate of CO\(_2\) evolution expressed per square cm of leaf surface per hour was a value directly applicable to results obtained for the respiration of the living host-pathogen complex.

The results are presented in Table 6.

The difference between the mean respiration rates of the lower and upper leaves is not significant at \( P = 0.05 \). It was assumed from these results that a respiration rate of \( 1.751 \text{ mm}^3 \text{CO}_2 \text{ cm}^{-2} \text{hr}^{-1} \), was a reasonable figure to cover the respiration due to the fungus in a host-fungus combination of \( \text{P. eucalypti} \) on \( \text{E. bicostata} \).

Certain objections could be made to the use of this procedure for computing the respiration due to the fungus in the pathogen living leaf complex. The method assumes that the substrates present in a killed leaf are as satisfactory for the respiration by \( \text{P. eucalypti} \) as are the substrates of a living leaf and that the organism penetrates a dead leaf and respires the substrates available in it at approximately
the same rate as it penetrates and respites the substrates of a living leaf. These assumptions would require considerable experimental work for substantiation. However the figure suggested is the respiration from a leaf area which can be considered as permeated 100% by the fungus. The infected leaves used in the gas exchange experiments on living host pathogen complexes never had more than 50% of their total area lesioned at the time of measurement, and generally, fungus was present in only 30% - 40% of the total leaf area. In the killed leaves the fungal mycelium spread through the palisade tissue to the same degree as it did in the spongy mesophyll. In the infected living leaves most of the fungal growth was in the spongy mesophyll with limited spread into the palisade tissue i.e., degree of penetration of the living leaves was less in both area and depth, than the penetration of dead leaves. Taking these factors into account the figure suggested is probably rather excessive for assessing fungal respiration.

An alternative method was attempted by assessing the respiration rate of a large piece of fungus culture - 8.8 mgms dry weight - scraped from the surface of a monospore sterile culture on malt-agar. This piece of culture did not produce a measurable deflection in the analyser. This is understandable since the area of surface exposed in such a culture is relatively small and the rate of respiration would be severely
restricted by the rate of movement of CO₂ from the inside to the outside of the culture.

Observations on effects of certain factors on respiration and diffusion rate of fungus and leaves

Several points of interest were noted with the respiration of the artificially infected dead leaves described above. When the lights in the growth cabinet were switched on to full light intensity the "respiration" of the fungus penetrated, dead leaves increased considerably. The respiration of four such leaves was measured in the dark and in the light. The mean respiration measurement in the light was almost 3 times that in the dark and the difference using the four replicates was significant at P < 0.001. This indicates that light has a significant effect on the "respiration" of such leaves as measured with an I.R.G.A. This matter was not further investigated.

Views on the relative levels of light and dark respiration of higher plants are rather contradictory (Brown, 1953; Decker, 1959; Treguna, Krotkov and Nelson, 1961). No data was available for the effect of light on respiration of these fungi. In this instance it would be important to establish that the increased "respiration" in the light was really due to increased CO₂ production and not to the production of some other gas which had a similar absorption spectrum to CO₂.
In some of the preliminary experiments measuring the respiration rate of living infected and uninfected leaves, it was difficult to obtain a stable zero on the I.R.G.A. (Work was in progress in the Botany Dept. basement at the time). It was decided to remove the CO$_2$ from the incoming air stream with Carbosorb and use this CO$_2$ free air to circulate over the leaf and through the reference side of the analyser. Using this air source it was possible to obtain a stable zero very quickly. When this variation in the circuit was tested on a number of plants of both *E. bicostata* and *E. tereticornis*, it was found that the respiration as measured by the I.R.G.A. was some 3-4 times higher when using CO$_2$ free air than when using normal basement air containing 0.031% CO$_2$. A reduction in the CO$_2$ of the surrounding environment could reasonably affect the rate at which CO$_2$ is exchanged between the leaf and the environment. (The diffusion gradient from the leaf is steeper as the CO$_2$ content of the air surrounding the leaf is reduced.) However, on the theoretical equations postulated to control such diffusion exchanges, this increase should be merely temporary (Gaastra, 1959; Bierhuizen and Slatyer, 1964). In the instances examined the higher rate was maintained steady for over 1½ hours. This suggests that the CO$_2$ free air in the environment surrounding the leaf
probably has a very significant effect on one of the resistances interposed between the centre of respiration and the outside of the leaf. This matter was not further investigated but it has significance in investigations of absolute rates of respiration, will be lower than that calculated from

6. 44 Significance of Leaks in the Circulation System in the rates of Photosynthesis and respiration recorded. Leaks. It will be appreciated that it is very difficult in a complex circulation system to have the system free of leaks. It has been claimed that since such leaks will be outward, providing they are only small, they are of little significance, (Gaastra, 1959). Evidence accumulated in the preliminary work that this is only partly true. Leaks which occur between the vertical flow metres - where the rate of flow is determined in litres per hour - and the leaf chambers are very important. If any air is lost in this portion of the circuit, the rate of apparent photosynthesis or respiration will be exaggerated, because of the formula used to calculate these values. The rate of gas exchange is calculated from

\[ \text{Rate} = \frac{D \times 0.075 \times \text{Flow Rate}}{\text{Leaf Area}} \]

Theoretically, Flow Rate in this formula means the flow rate in the leaf chamber where the gas exchange is occurring, in practice the flow rate is determined at the vertical flow.
metres of some distance from the leaf chamber. If there is a leak between the flow metres and the chamber, the actual flow rate in the chamber will be lower than that used in the formula (determined from the flow metres). Thus the true rate of gas exchange will be lower than that calculated from the formula. Even a very small leak in this region will be very important in inflating the apparent gas exchange rate. Leaks before the vertical flow metres or after the leaf chambers are of no significance. Leaks after the leaf chamber merely allow the loss of a sample of air which is assumed to have reached the CO₂ equilibrium of the leaf chamber environment. In the open system used in these experiments, only 15 litres per hour of air was passed to the analyser irrespective of whether the flow rate in the leaf chamber was 35 L or 150 L per hour. From the point of view of the final gas analysis figure obtained, much less than 15 L per hour could be passed through the analyser, providing the same quantity passed through both the analysis and reference sides of the machine. The only difficulty introduced here with lower flow rates is the time taken for the analyser to stabilise at the particular CO₂ deflection difference if the flow rate is very low e.g. 8 litres per hour.

6.50 Discussion

Improvements which could be made in the instrumentation
to improve its efficiency are:

1. The installation of a more satisfactory system for freezing out the water from the gas stream after it passes the horizontal manometers and before it passes to the analyser. The present system of using water traps touching ice-alcohol mixtures in thermos flasks is time consuming to set up. The ice alcohol mixture - even if the alcohol is kept in a cold room - takes approximately 1 hour to reach a temperature of -20°C. As the ice melts, the level of liquid in thermos flask rises and drops of moisture form ice on the outlet sides of the water traps. These can close off the supply of air to the I.R.G.A. Their removal once again means loss of time. The ice alcohol mixture only remains at a temperature of -20°C while there is unmelted ice in the flask, after this it gradually rises and the removal of water becomes less efficient.

An improvement would be the installation of a refrigeration system with external freezing coils which could maintain a temperature of -20°C continuously. This, combined with a system of interchangeable traps, should reduce the setting up time considerably. Improvements could also be made in the trap design, by the use of internal baffles all moisture could be frozen out of the air stream before it reached the outlet pipe of the trap.
2. The installation of flow metres on the air lines after these have passed through the leaf chamber and the blank chamber respectively is very necessary. This would ensure that there were no leaks in the system which could significantly affect the calculated rate of gas exchange. In this particular set of experiments small leaks in this portion of the circuit were probably of less importance because the study was a comparative one. In addition because the measurements were replicated (by 12 leaves at each time of measurement and also in time (i.e., by successive measurements) inconsistencies were readily noticeable. In individual observations where absolute values of gas exchange were being determined, the installation of these extra flow metres would be essential.

3. A further improvement suggested would eliminate to a considerable degree the irregular traces obtained on the chart when the CO₂ content of the incoming air varies to a small degree. The I.R.G.A. records the difference in concentration between the CO₂ contents of the leaf chamber and blank circuits of the analyser. Theoretically small variations in the CO₂ content of the incoming airstream should have little or no effect on this differential. This assumes that the volume of air between the mixing flask and the analyser is identical in both the
leaf chamber and blank circuits. In the setup used these two lines were not absolutely identical in volume although the volume of the blank chamber had been adjusted with glass beads to the approximate volume of the leaf chamber. If the volumes of the two circuits were identical then the differential measured would only be varied by effects which the changed CO₂ content had on the particular physiological process being recorded.

This last effect could be eliminated by following the procedure used by certain workers of removing the CO₂ from the incoming air and then adding a measured quantity of CO₂ to the air stream before it passed to the leaf chamber, (Bierhuizen and Slatyer, 1964).

The results of transpiration measurement, using the wet and dry thermocouple psychrometers, were not satisfactory. In general throughout the experimental period the trend of transpiration as calculated from the wet and dry bulb readings (Bierhuyzen and Slatyer, 1964), followed that of photosynthesis. This is in agreement with the results of other workers (Bosian, 1963). However when the leaf area of infected leaves was reduced by the necrosis of invaded tissue, there were frequently no measurable changes in transpiration rate. Also uninfected leaves with differences of 10-15% in area, frequently showed
identical total calculated, transpiration. Probably the reason for these results is the accuracy to which the record chart can be read. .25 units on the recorder chart represents 1.7°C. It is impossible to read the chart to a greater accuracy than .25 of a unit and this accuracy is not sufficient for meaningful results. The psychometers and recorders used by other workers gave a chart reading to the nearest millimetre and each unit was equal to .127°C (Bierhuizen and Slatyer, 1964).

Because of the above, measurements of the time course transpiration are not presented. It would seem desirable in any future work of this kind to use an I.R.G.A. for recording the transpiration. Ample air was bled off at the horizontal manometers to supply a second I.R.G.A. used for this purpose. Other workers have adapted the I.R.G.A. for measurement of transpiration (Decker and Wetzer, 1957; Decker and Wien, 1960) and the accuracy of these instruments should enable satisfactory results.

Conclusions

1. The saturation light intensity for apparent photosynthesis in E. tereticornis is $8.8 \times 10^6$ ergs per sq cm per sec and for E. bicostata $11.1 \times 10^6$ ergs per sq cm per sec.

2. The mean respiration rate of P. eucalypti on artificially inoculated dead leaves is $-751$ mm$^3$ CO$_2$ per sq cm of infected killed leaf of E. bicostata per hour.
CHAPTER VII

Time course of Photosynthesis in leaves of eucalypt species infected with Phaeoceptoria eucalypti compared with that in uninfected leaves on the same plants.

7.1 Introduction

The materials and instrumentation used in this study, have already been given in Chapter 6.

The plants for the first experiment using E. tereticornis were raised from seed in the Ceres phytotron. As outlined previously the seed was germinated in petri dishes the seedlings were transferred to 5" pots of perlite at three weeks. After planting out the seedlings were placed in the 27°C phototemperature, 22°C nyctotemperature glasshouse. At five weeks the seedlings were thinned to one per pot. At ten weeks the seedlings had eight pairs of approximately opposite fully expanded leaves. It was decided to use the third lowest leaf pair on six apparently comparable plants for gas exchange measurements. The tops of the plants above this pair of leaves were cut off and thereafter all shoots were bud pruned.
so that no shading of the leaves used for measurement occurred. The outline of each selected leaf on each plant was drawn and its area computed by planimeter.

One of the third pair of leaves on each of the six plants was marked and inoculated with a heavy spore solution of *P. eucalypti* on three successive days. During the inoculation period the plants were kept in a glass fronted incubation cabinet at a high humidity, at a temperature of 25°C and with supplementary light supplied from two Condor incandescent lamps.

7.2 Experiment 29 Apparent photosynthesis of infected and uninfected leaves of *E. tereticornis*.

After four days incubation the plants were transferred to the growth cabinet. The infected and uninfected leaf of each third leaf pair on the six plants were then placed in separate leaf chambers as described previously. When this setting up was completed the general air circulation supplied by a small reciprocating air pump delivering 2000 litres per hour was turned on and the leaves allowed to settle down in the new environment for two days.

The setting up procedure was not entirely satisfactory. Silastic rubber was used to seal around the petioles and there were small leaks in this which had to be repaired. In addition one member of each leaf pair on two plants was broken off
during setting up. This occurred because the leaves were not exactly opposite and the leaf chambers were too rigid in their relative height adjustment. The general air circulation from the small air pump was not reliable. As a result, the air circulation over the leaves ceased and the chambers fogged up. Following these breakdowns, dead areas appeared on a portion of one of the leaf pairs of another of three plants. This dead area reduced the photosynthetic area of the leaf and consequently affected the rate of apparent photosynthesis.

thermocouples. As indicated in Chapter 6, this experiment provided valuable experience in the use of this equipment and in recognising the difficulties inherent in the experimental arrangement. The only result of significance in relation to the measurement of the time course of photosynthesis, was that in plants, where a comparison could be made, there was an
obvious rise in apparent photosynthesis of both infected and uninfected leaves some 19-23 days after inoculation.

It was also apparent, from an examination of the wet and dry bulb temperature readings on leaves which developed dead areas, that the wet and dry bulb psychrometers were not sufficiently accurate to show up differences in the transpiration of infected and uninfected leaves, if such differences were present. Despite the death of small areas on leaves, there was no measurable change in the transpiration of the leaves as indicated by the wet and dry bulb recordings of the psychrometer thermocouples. As indicated in Chapter 6, this was probably due to the level of accuracy with which differences in transpiration could be recorded.

It was decided as a result of the difficulties in setting up to make several changes. The first of these was to use modelling clay instead of silastic rubber as the sealing compound. A further change was to use a species with directly opposite leaves - *E. bicostata* for the second experiment. Finally, a more reliable general air circulation system was introduced. This involved the tapping of a piped compressed air supply. The tap on this supply had a needle valve thus the rate of air flow could be carefully adjusted and maintained in a fixed position.
7.3 Experiment 30  Apparent photosynthesis of infected
and uninfected leaves of E. bicostata

For this experiment plants of E. bicostata were raised
in the Ceres phytotron at a photo temperature of 27°C and
nycotemperature of 22°C as described previously. At ten
weeks, six plants were selected which had approximately
cul-
uniform undamaged pairs of leaves at the third lowest leaf
node. The juvenile leaves of E. bicostata are sessile.

Since they were to be fitted into the leaf chambers, while still
attached to the plant, it was necessary to modify the base of
the leaves. The lamina at the base of each leaf was carefully
cut to the midrib and removed to obtain a length of clear
midrib to stretch from the plant, through the hole for the
petiole and into the leaf chamber. After this cutting the
plants were allowed a further two weeks to recover.

At twelve weeks from planting the six plants were taken
from the phytotron and the tops, above the third lowest leaf
pair, removed. The outline of each leaf, to be used for
measurement, was traced and its area determined by planimeter.

Thereafter, the plants were bud pruned to prevent shading
of the lower leaves. All leaves on the plants apart from
one of the third leaf pair on each plant were inoculated
with a heavy spore suspension of P. eucalypti by a camel
hair brush, on three successive days. The leaves inoculated,
apart from those to be used for gas exchange measurement, six
were sampled for histological examination of the progress of
the infection (Chapter 1). Since these leaves would be in
approximately the same environment as those in the leaf
chambers, the progress of infection on them should be repres­
entative of those in the leaf chambers. During the inocul­
ation period and for two days afterwards the plants were
kept in the incubation cabinet at 25°C, with supplementary
lighting. Plants flooded with half strength Hoagland's solution.

After the incubation period the plants were transferred
to the growth cabinet, the leaves for gas exchange measurement
on each of the six plants were placed in separate leaf
chambers. The chambers were sealed with modelling clay. The
plants were allowed 48 hours to equilibrate to the new envi­
ronment. The general circulation was from the compressed air
supply tap. The rate of flow from this tap was adjusted so
that at full light intensity there was no fogging of the leaf
chambers. The flow rate over the leaf was approximately 260
litres per hour. and uninfected leaves on three plants had
been After 48 hours, measurements of apparent photosynthesis
were commenced at full cabinet lighting (this varied from
11.9 to 11.7 ergs cm \(^{-2}\) sec \(^{-1}\) over the period of the experiment -
48 days). The measurements of apparent photosynthesis were
alternated daily with those of respiration. The measurement
of apparent photosynthesis for the 12 leaves usually took six hours, those of respiration seven hours. The longer period for the respiration measurements was due to the sluggishness of the I.R.G.A. to settle to a stable reading at the flow rate of 35 litres per hour. Apparent photosynthesis was measured using a flow rate of 150 litres per hour. The sequence of procedures for measurement was:

8 a.m. The fluorescent lights on in the cabinet.
9 a.m. Plants flooded with half strength Hoagland's solution.
9.30 a.m. Recordings commenced on the I.R.G.A., with leaf chambers and reference blank bypassed, to obtain a stable zero.
10 a.m. Measurements of apparent photosynthesis commenced.

The recording of gas exchange of each chamber was continued until a steady trace was obtained for ten mins. The chamber was turned off and the opposite leaf chamber on that plant cut in and measurements recommenced.

When the gas exchange for six leaf chambers i.e., the pairs of infected and uninfected leaves on three plants had been recorded, the chambers and reference blank were bypassed and the I.R.G.A. re-zeroed. When a stable zero had been obtained measurements of the gas exchange of the other six leaf chambers were made.
TABLE 7. Sample data of rate of apparent photosynthesis at successive measuring times on several plants.

<table>
<thead>
<tr>
<th>Infected leaves</th>
<th>Apparent rate of Photosynthesis in ( \text{mm}^3 \text{CO}_2 \text{cm}^{-2} \text{hr}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant No.</td>
<td>Date</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
4:00 p.m. Measurements completed and final zero taken with I.R.G.A.

4:30 p.m. Plants flooded with demineralised water.

The recordings of gas exchange in recorder units were converted to apparent photosynthesis rate in $\text{mm}^3 \text{CO}_2 \text{cm}^{-2} \text{hr}^{-1}$ for each leaf, by the formula

$$P = D \times 0.275 \times \frac{\text{Flow rate}}{\text{Leaf area}}$$

where $P =$ apparent photosynthesis and $D =$ deflection in recorder units, Flow rate determined in litres per hour at the vertical manometers and leaf area in $\text{sqr cms}$, from planimeter measurements.

It was evident, after the first three days of measurements, that while the absolute rates of apparent photosynthesis of individual plants were reasonably reproducible from day to day, there were considerable differences in the absolute rates of different leaves. This is illustrated in Table 7.

This high degree of variability between individual plants, which occurred in both infected and uninfected leaves, would have made it impossible to achieve statistically significant differences between the infected and uninfected treatment. This within treatments variability was eliminated by calculating the mean of the absolute rates of apparent
TABLE 7.3.2 Mean rate of apparent photosynthesis of infected and uninfected leaves of *E. bicostata* expressed as a percentage of the initial rate.

<table>
<thead>
<tr>
<th>Days after final Inoculation</th>
<th>Leaves infected with <em>P. eucalypti</em> as % of initial rate</th>
<th>Uninfected Leaves as % of initial rate</th>
<th>Difference (Uninfected - infected)</th>
<th>Variance Ratio*</th>
<th>Significance at P = .05</th>
<th>Statistical Test used and significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of days 6, 8 @ 9</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>-</td>
<td>N/S</td>
<td>Student T test N/S</td>
</tr>
<tr>
<td>10</td>
<td>103.1 ± 9.7</td>
<td>102.3 ± 9.1</td>
<td>- 0.8</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S</td>
</tr>
<tr>
<td>13</td>
<td>95.5 ± 4.0</td>
<td>97.9 ± 1.95</td>
<td>+ 2.4</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S</td>
</tr>
<tr>
<td>17</td>
<td>89.2 ± 1.67</td>
<td>92.3 ± 2.81</td>
<td>+ 4.1</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S</td>
</tr>
<tr>
<td>19</td>
<td>86.8 ± 1.84</td>
<td>90.5 ± 1.60</td>
<td>+ 3.7</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S</td>
</tr>
<tr>
<td>24</td>
<td>115.4 ± 1.76</td>
<td>118.4 ± 1.61</td>
<td>+ 3.0</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S</td>
</tr>
<tr>
<td>26</td>
<td>114.3 ± 1.84</td>
<td>122.3 ± 2.31</td>
<td>+ 8.0</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S Sig @ P &lt; .05</td>
</tr>
<tr>
<td>31</td>
<td>116.0 ± 2.70</td>
<td>127.5 ± 3.53</td>
<td>+ 11.5</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S Sig @ P &lt; .05</td>
</tr>
<tr>
<td>38</td>
<td>106.5 ± 5.06</td>
<td>127.5 ± 3.50</td>
<td>+ 21.0</td>
<td>N/S</td>
<td>&quot; &quot; N/S</td>
<td>&quot; &quot; N/S Sig @ P &lt; .05</td>
</tr>
<tr>
<td>49</td>
<td>74.4 ± 15.76</td>
<td>121.0 ± 1.60</td>
<td>+ 46.6</td>
<td>Significant</td>
<td>Behrens T test N/S</td>
<td>Student T test Sig @ P &lt; .05</td>
</tr>
<tr>
<td>59</td>
<td>47.5 ± 7.65</td>
<td>113.9 ± 4.40</td>
<td>+ 66.4</td>
<td>N/S</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results are plotted in Fig 7.3.2.

*From Variance Ratio Tables for Significance at 5% level Fisher and Yates, 1948.*
photosynthesis on three successive days for each individual leaf. This mean for each leaf was equated to 100%. The readings taken thereafter for that leaf were expressed as a percentage of this initial rate of 100%.

The results of the measurements of apparent rate of photosynthesis are presented in Table 7.3.2. and Fig. 7.3.2.

7.3.1 General discussion

These results confirm the fragmentary evidence of the experiment using *E. tereticornis* seedlings. The apparent photosynthetic rates of both infected and uninfected leaves, on the same plant, rise some 19-24 days after the leaves on the plant are inoculated.

It is fairly clear from the gradual fall in photosynthetic rate in both infected and uninfected plants between 9 and 19 days (this fall of 10-15% is Sig at P< .01) after inoculation that the progress of photosynthesis with age, is what would be expected, from the data of other workers, on fully expanded leaves (Richardson, 1957; Clark, 1961; Freeland, 1952; Loustalot and Hamilton, 1941).

The rise in apparent photosynthetic rate at 10 days in both infected and uninfected by comparison with the initial rate is not significant. It should be noted also that 10 days was the only sampling time when the apparent photosynthesis of infected leaves exceeded that of uninfected leaves.
FIGURE 7. Time course of photosynthesis in infected and uninfected leaves of *P. biscopteta.*
an apparent rate of photosynthesis as a percentage of initial rate.

Time after inoculation (days)
This slight non-dignificant difference between infected and uninfected leaves could nevertheless be the end point of the excitation of photosynthesis following inoculation noted by other workers for wheat leaves infected with powdery mildew (Allen, 1942; Sempio, 1950a). The most obvious interesting point in the results is the very considerable (15% - 20% Sig in both cases at P < .001) rise which occurs in apparent photosynthetic rate 24 days after inoculation. The rise occurs in both infected and uninfected leaves. Following this rise the rates of apparent photosynthesis in the two treatments differ. The rate of apparent photosynthesis in the infected leaves falls much more quickly than that in the uninfected leaves. By 59 days the apparent photosynthesis of the infected leaves is only 47% of the initial rate and is less than 50% of the apparent photosynthetic rate in uninfected leaves.

If the standard errors of the mean rate of apparent photosynthesis for the infected leaves are examined it is obvious that all infected leaves have not shown the same degree of reduction of apparent photosynthetic rate with time after the initial increase at 24 days. The very large standard error of the mean of infected leaves at 49 days indicates that some infected leaves still have a relatively high apparent photosynthetic rate at this stage. (Results
for individual leaves shows variations from 55% to 100% of the initial rate. The mean apparent photosynthetic rates of infected and uninfected leaves are not significantly different at this time because of this large standard error. A reasonable explanation of the obvious differences in the degrees of standard error in the means of infected and uninfected leaves is that the infected leaves on different plants are at different stages in their photosynthetic reaction at the same time. This difference can be explained on either different degrees of infection or different degrees or times of reaction of the particular host leaf to infection. It would be expected that if genetically uniform plants were used and they were uniformly inoculated, they would show a more uniform reaction in apparent photosynthesis at a particular time after inoculation.

Histological observations were carried out on other inoculated leaves on these plants (Chapter 1). At 23 days after inoculation when the inoculated leaves were cleared, although before clearing there was no superficial evidence of infection, areas smaller in diameter than the interveinal islands of the leaf failed to clear completely in 95% methanol (Chapter 1). As pointed out these "green" areas are the first microscopic evidence of infection. As the infection progresses the "green" areas enlarged to occupy the whole of the interveinal...
island areas around infection courts. Thus the rise in apparent photosynthesis rate in infected and uninfected leaves corresponded with the time of production of the first histological reaction in infected leaves.

The rise in apparent photosynthesis in both infected and uninfected leaves on infected plants, agrees with observations on bean leaves lightly infected with bean rust, when a radioactive isotope technique was used for measurement of photosynthesis (Livne, 1964). The sharp decline in apparent photosynthesis after 31 days in infected leaves corresponded to the period of pycnidial production culminating in extensive sporulation. This phenomenon of a sharp decline in photosynthesis at the time of sporulation has also been noted (Livne, 1964). It is in contrast to some data for powdery mildew on wheat where the period of maximum photosynthesis in infected leaves corresponded to the time of formation of conidiophores and conidia and still later at the time of abundant conidia production (Sempio, 1946, 1950a).

Unfortunately Livne's (1964) paper was not available at the time when this experiment was set up. His observations indicate that uninfected leaves on infected plants are not a satisfactory "control" for the comparison of the effect of infection on the rate of apparent photosynthesis. Comparable leaves on uninfected plants would provide the only satisfactory
"control" to observe the comparative progress of apparent photosynthesis in infected and uninfected leaves with time. Despite this shortcoming the results are fairly conclusive. The rise in apparent photosynthesis at 24 days after inoculation is abnormal in the time progress of apparent photosynthesis in normal leaves as they age. The most reasonable conclusion is that this rise is a result of the inoculation of the plants. This is particularly so if account is taken of Livne's results.

The data on photosynthesis presented here also that of Sempio (1950) and that of Livne (1964) are in apparent conflict with that for papaya leaves infected with leaf mosaic (Decker and Tio, 1958) which was performed with an E.R.E.A. and also with that for downy spot on pecan leaves (Loustalot and Hamilton, 1941) carried out using standard analysis techniques. This conflict is not surprising since in neither of the latter cases mentioned was the study of apparent photosynthesis carried on throughout the infection period of the parasite. The papaya leaves were inoculated in December 6th and the photosynthesis of infected and control leaves measured on February 5th-7th. The photosynthetic measurements on infected and uninfected pecan leaves were commenced only when symptoms of the disease were apparent. The conflict with the work using powdery mildew on wheat
(Allen, 1942) is more real but Sempio's results (1959) using the same host parasite combination as Allen, show the same conflict (Chapter 6).

The results suggest some very interesting possibilities which are capable of experimental investigation. The rise in apparent photosynthesis of uninfected leaves on infected plants as a result of infection suggests that infection of leaves by \( P. \) eucalypti (also by bean rust in the case of Livne's (1964) work) causes a systemic response in the other leaves of the plant. Systemic responses expressed by increased disease resistance of leaves following stem infection of tobacco by \( P. \) tabacina Adam, have been reported (Mandryk, pers.com). It seems reasonable to postulate that following infection of the leaf of \( E. \) bicostata by \( P. \) eucalypti, a material is produced in the infected leaf which causes a rise in photosynthesis. This material is presumably transported to the opposite uninfected leaf where a rise in apparent photosynthesis also occurs.

A radio active tracer technique is the logical means by which to investigate this possibility. If \( ^{14}C_2 \) were fed selectively to an infected leaf, while the opposite uninfected leaf received normal \( CO_2 \), and radio active material appeared in the uninfected leaf, then this would be good prima facie evidence that the proposed mechanism occurred. The approach
to the biochemical side of the problem would depend on the result of this initial investigation. In the present results there is some support for this suggestion. The large standard errors of the means of apparent photosynthesis in the infected leaves after 38 days are due to great differences between apparent photosynthesis in individual infected leaves. The apparent photosynthesis in an infected leaf will depend on the balance between the postulated promoter of photosynthesis and the reduction occurring in photosynthesis due to death of the host tissue following infection. By contrast in the uninfected leaves if only the postulated promoter of photosynthesis is active then standard errors of the means of apparent photosynthesis in such leaves would be expected to be smaller and also the promoter effect would be expected to last longer. Just this situation in regard to the degree of standard error and the period over which the significant promotion of apparent photosynthesis occurs is evident in the results. The relationship between these results and those obtained for the time course of respiration will be discussed at the conclusion of the following Chapter.

7. Conclusions

Following inoculation of leaves of *E. bicostata* with *P. eucalypti*,

1. The time course of apparent photosynthesis in both infected and uninfected leaves shows a steady fall for 19 days after inoculation. The rate after 19 days is only 86% - 90% of the initial rate (difference is Sig at P < .01).

2. At 24 days after inoculation the apparent photosynthesis of both infected and uninfected leaves shows a considerable significant rise. A rise of 15% - 20% (Sig at P < .001).

3. The rate of apparent photosynthesis in uninfected leaves fell slowly after 31 days and at 59 days is still above its initial rate. By contrast the rate of apparent photosynthesis in infected leaves falls as the organism produces pycnidia and commences to sporulate. At 59 days the mean apparent photosynthetic rate of infected leaves is less than 50% of the initial rate.

E. tereticornis 2 months old which had been raised in the Ceres phytotron glasshouse (phototemperature 27°C nycotemperature 22°C). One leaf of each of the 2nd lowest leaf pair on the 4 plants was inoculated with a heavy spore suspension by a camel hair brush. The plants were incubated in the incubation chamber at 25°C with supplementary lighting for 4 days. At 30 days after inoculation, the plants had meanwhile been kept in the growth cabinet, chlorotic areas were evident on the
CHAPTER VIII

Time Course of Respiration in leaves of Eucalyptus infected with P. eucalypti compared with that in uninfected leaves on the same plant.

The materials and instrumentation used in these experiments have been described in Chapters 6 and 7.

8.1 Experiment 37 Preliminary investigation of the respiration level in infected and uninfected leaves of E. tereticornis on the same plants at 30 days after inoculation.

A preliminary experiment was conducted using plants of E. tereticornis 2 months old which had been raised in the Ceres phytotron glasshouse (phototemperature 27°C nycotemperature 22°C). One leaf of each of the 2nd lowest leaf pair on the 4 plants was inoculated with a heavy spore suspension by a camel hair brush. The plants were incubated in the incubation chamber at 25°C with supplementary lighting for 4 days. At 30 days after inoculation, the plants had meanwhile been kept in the growth cabinet, chlorotic areas were evident on the
TABLE 8. Respiration rates of infected and uninfected leaves of *E. tereticornis* (One leaf pair, one leaf infected with *P. eucalypti* and one uninfected, was taken on each of four plants).

Mean respiration rate in mm$^3$ CO$_2$ cm$^{-2}$ hr$^{-1}$

<table>
<thead>
<tr>
<th>Infected leaves</th>
<th>Uninfected leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.56 ± 0.50</td>
<td>3.95 ± 0.03</td>
</tr>
</tbody>
</table>
infected leaves and some fruits of *Eucalyptus* were forming. The respiration rates in \( \text{mm}^3 \text{CO}_2 \text{cm}^{-2} \text{hr}^{-1} \) were measured on each of the four inoculated and opposite uninoculated leaves, using a flow rate of 35 L per hour. As explained in Chapter 7, the results are presented in Table 8.1. The difference between the means of 40% is significant at \( P < 0.05 \). This preliminary experiment was conducted as a leader test to indicate the approximate time at which an increase in the respiration rate of infected leaves could be expected and the extent of the increase. The flow rate used for this experiment indicated that while the respiration rates of opposite leaves on the same plant were comparable, the respiration of infected leaves on different plants showed considerable variation. It was decided in the following major experiments on the time course of respiration to convert the absolute readings of respiration to a percentage value of the initial rate for the individual infected and uninoculated leaves.

**8.2 Experiment 39: Time Course of Respiration in Infected and Uninfected Leaves on *E. tereticornis* Plants**

As was the case with the data for Expt. 29, in the time course of photosynthesis the difficulties in setting up the experimental material and the break-down in the air pump on the general circulation to the leaf chambers made the exper-
imental results largely valueless. In the leaves which survived these difficulties, there was a pronounced rise (some 35%) in the respiration of infected leaves by comparison with opposite uninoculated leaves. As explained in Chapter 7 this experiment on the time course of change in physiological functions was repeated using E. bicostata.

8. 30 Experiment 59 Time course of respiration in infected and uninoculated leaves on E. bicostata plants.

The setting up of, and the materials used for, this Experiment have been described in Expt. 56. The flow rate used throughout was 35 litres per hour. The absolute respiration rates measured on the 7th, 10th and 12th days after the final inoculation of the leaves were averaged and this value was taken as 100% for each leaf (i.e., the "initial" value of respiration). Measurements of respiration at succeeding days after inoculation were expressed as a percentage of this initial rate.

As explained in Chapter 7 the normal procedure was to measure apparent photosynthesis one day and respiration the next. The normal light regime between successive respiration measurements was:

Measurement of Respiration - Darkness finish 4.30 p.m.,
4.30 p.m. - 8 p.m. - Photoperiod, incandescent lights
8 p.m. - 4 a.m. - Darkness
**TABLE 8.3**

Respiration rate of Infected and Uninfected leaves of *E. bicostata*. Respiration expressed as a percentage of the initial rate.

<table>
<thead>
<tr>
<th>Days after Inoculation</th>
<th>Respiration Infected leaves as % of initial rate</th>
<th>Respiration Uninfected leaves as % of initial rate</th>
<th>Difference Infected-Uninfected</th>
<th>Variance Ratio</th>
<th>Significance of Difference and Test used.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>102.2 ± 5.47</td>
<td>100.6 ± 1.29</td>
<td>+ 1.6</td>
<td>15.742 Sig.</td>
<td>N/S Behrens T. test</td>
</tr>
<tr>
<td>20</td>
<td>102.7 ± 2.36</td>
<td>106.1 ± 3.29</td>
<td>- 3.4</td>
<td>2.321 N/S</td>
<td>N/S T. test</td>
</tr>
<tr>
<td>22</td>
<td>89.2 ± 2.54</td>
<td>97.5 ± 3.53</td>
<td>- 8.3</td>
<td>4.880 N/S</td>
<td>N/S T. test</td>
</tr>
<tr>
<td>29</td>
<td>107.8 ± 3.79</td>
<td>107.6 ± 7.05</td>
<td>+ 0.2</td>
<td>2.764 N/S</td>
<td>N/S T. test</td>
</tr>
<tr>
<td>32</td>
<td>144.5 ± 4.85</td>
<td>112.6 ± 4.46</td>
<td>+31.9</td>
<td>1.204 N/S</td>
<td>N/S T. test</td>
</tr>
<tr>
<td>36</td>
<td>146.0 ± 6.20</td>
<td>111.4 ± 3.0</td>
<td>+34.6</td>
<td>4.289 N/S</td>
<td>Sig. @ P&lt;.01 T. test</td>
</tr>
<tr>
<td>39</td>
<td>148.6 ± 11.34</td>
<td>114.9 ± 2.56</td>
<td>+33.7</td>
<td>19.545 Sig.</td>
<td>Sig. @ P&lt;.05 Behrens T. test</td>
</tr>
<tr>
<td>46</td>
<td>148.7 ± 15.09</td>
<td>118.6 ± 2.30</td>
<td>+30.1</td>
<td>30.892 Sig.</td>
<td>N/S Behrens T. test</td>
</tr>
<tr>
<td>51</td>
<td>148.9 ± 7.74</td>
<td>119.7 ± 3.8</td>
<td>+29.2</td>
<td>20.0 N/S</td>
<td>Sig. @ P&lt;.05 T. test</td>
</tr>
<tr>
<td>59</td>
<td>135.9 ± 2.24</td>
<td>124.5 ± 3.2</td>
<td>+7.4</td>
<td>4.72 N/S</td>
<td>Sig. @ P&lt;.05 T. test</td>
</tr>
</tbody>
</table>
Measurement of Respiration - Darkness finish 4.30 p.m. (contd)

4 a.m. - 8 a.m. - Photoperiod incandescent lights
8 a.m. - 4 p.m. - Fluorescent lights - measurement of apparent photosynthesis
4 p.m. - 8 p.m. - Photoperiod incandescent lights
8 p.m. - 4 a.m. - Darkness
4 a.m. - 8 a.m. - Photoperiod incandescent lights
8 a.m. - 9 a.m. - Fluorescent lights
9 a.m. - 11 a.m. - Darkness
11 a.m. - Commence Respiration Measurements

On one occasion during the measurements, it was decided to vary this regime and the 8 hours fluorescent lighting the previous day was cut out i.e. on the 21st day after the final inoculation. The aim of this variation was to observe the effect of the previous light regime on the respiration in the following dark period.

The results of this experiment on the time course of Respiration are presented in Table 2, 3 1.

The results are plotted in Figure 8, 3 1.

It is evident from these results, that there has been a very significant increase in the respiration of infected leaves, during the period 29-50 days after inoculation by comparison with the opposite uninfected leaves. It is also evident that during this period infected leaves on different
FIGURE 8, 31.

Time Course of Respiration of leaves infected and uninfected leaves of *E. bicostata*. Respiration rate expressed as a percentage of the initial rate.
Mean rate of Respiration as a percentage of initial rate

Time after inoculation (dys.)
plants have not all reached their maximum respiration on the same date (the great differences in the standard error of the means of respiration rate of infected plants on the 32nd, 36th, 39th and 46th days). A comparison was also made between the maximum respiration rates achieved by individual infected leaves and the corresponding respiration rate of uninfected leaves on the same date (referred to as the mean maximum respiration rate.)

The difference between the mean maximum respiration rate of infected leaves and the mean rate of respiration of uninfected leaves on the same dates is 36.3%. This difference using Student's T-test is significant at P<.001.

The respiration rate of the fungus alone, on killed leaves of E. bicostata was determined as 0.75 mm³ cm⁻² hr⁻¹ (Expt 28). This value was added to each of the absolute values of the respiration of uninfected leaves. These corrected levels of respiration of uninfected leaves plus fungal respiration were determined as a percentage of the initial rate of uninfected leaves. The mean respiration of uninfected leaves plus fungus is 133.1% of the initial rate of uninfected leaves alone. A comparison was made between the mean maximum respiration rate of infected leaves and the mean rate for uninfected leaves plus fungus on the same dates. This comparison shows a difference of 18.4%. This difference of means is significant.
at $P < 0.02$ (Student's $t$ test).

8. **General Discussion**

The results show that over the period of the experiment the uninfected leaves have shown a rise of some $24\%$ in mean respiration rate ($\text{Sig.} @ P < 0.001$). This rise in rate of respiration with increasing leaf age has been noted by other workers, (Ornrod, 1964; Clark, 1961). Not all workers agree that respiration rate increases with age (Kidd, Briggs and West, 1921; Geronimo and Beevers, 1964). Some of the apparent contradictions between results of different workers can be explained. Certain workers have used detached leaves. The significance of root growth in the level of respiration and photosynthesis in leaves has recently been emphasised (Humphries and Thorne, 1964). In other cases workers have compared leaves which differ in ontogenetic as well as physiological age and consequently changes in respiration rate cannot be satisfactorily correlated with either of these variables individually. The situation with regard to respiration of field crops has been summarised. "Respiration is not an independent process it is closely related to photosynthesis and physiological activity in different plant tissues" and again "In general very little is quantitatively known about the respiration rates of various plant organs throughout the ontogeny of field crops". (Gaastra, 1963).
The dependence of the dark respiration rate on the photosynthetic activity during the previous light period is also illustrated in the data. At 22 days after inoculation, when the plants received no illumination in the normal 8 hours photosynthetic period of the previous day, the respiration rate of both infected and uninfected leaves was significantly reduced. This result is in agreement with those of other workers emphasizing the importance of the previous light history of the organ in determining absolute dark respiration rate (Pisek, and Trancuillini, 1954).

Apart from quantitative changes in respiration it has been emphasised elsewhere that as a plant ages there is a change of predominance of catabolism from the glycolytic pathway to the hexose monophosphate pathway (Gibbs and Beevers, 1955). No attempt was made in this study to investigate qualitative changes in respiratory pathways.

It is interesting to note that the predominant participation of the hexose monophosphate pathway in infected plants may have a close connection with the biosynthesis of polyphenols and coumarin derivatives (Uritani and Akazawa, 1955). It has also been suggested that the capacity of the glycolytic pathway may not be sufficient to fulfill the total energy requirement of the host (Unitani and Akazawa, 1959). In uninfected leaves it would be expected that the maximum
energy requirements would be at the time of leaf expansion, and the explanation for the postulated change over may be in the hormonal control of respiration. The addition of certain plant growth hormones, causes a decrease in the C₆:₈ ratio (Humphreys and Dugger, 1957).

The most significant result in this study of the time course of respiration in infected and uninfected leaves is the higher level of respiration which occurs over the period 29-50 days after inoculation in the infected leaves. From histological studies this period corresponds to the time of fructification of the pathogen and tissue necrosis of the host i.e., the symptoms of disease become pronounced during this period of enhanced respiration. From the analysis of results it appears that the rise in respiration is contributed to about equally by the fungus and the host. This may not be quite correct since the respiration rate for the fungus was assessed on the basis of a fully infected leaf. As pointed out previously, in this time course experiment full infection of the host leaf was never attained, and it is likely that the actual rate of fungal respiration in the infected leaves is lower than that assumed.

The rise in respiration rate of 35%-40% following infection is relatively small by comparison with that observed by workers using host obligate parasite combinations where
differences of several hundred percent have been measured between infected and uninfected leaves (Sempio, 1959; Scott and Smillie, 1963). In the case of powdery mildew on susceptible wheat varieties, the first large rise in respiration occurs when there are numerous haustoria in the host cells and the mycelium of the pathogen is well developed. The maximum rise in respiration occurs when conidia are being produced in abundance and the attack is severe (Sempio, 1959). In the case of P. eucalypti on E. bicostata, the situation is slightly different. At 17 days after inoculation the hyphae of the pathogen had spread widely intercellularly and cushions of fungal tissue were forming in the substomatal cavities adjoining the stomata of penetration. These cushions are the precursors of pycnidia of the fungus. Up to this stage there was no significant rise in respiration of infected leaves by comparison with their uninfected counterparts. Necrosis was commencing in the leaf and the fungus was fruiting before the infected leaf showed any signs of increased respiration i.e., at 32 days. The increased respiration of infected leaves by comparison with uninfected ones continued for a further 16 days while more areas of the leaf necrosed, and the number of fruits of the pathogen increased. A comparison between the rise in apparent photosynthesis and respiration in the case of powdery mildew of wheat and the associated processes in the infected plant can provide valuable insights into the pathogen-host interactions.
changes in these processes in *P. eucalypti* on *E. bicostata* also shows certain differences (see Table 8.41).

In the case of powdery mildew of wheat on the 8th day after inoculation when the photosynthetic rise is at its maximum (apart from the initial excitation) the rate of respiration has increased nearly 300%, i.e., there is some evidence of a sympathetic movement of respiration with apparent photosynthesis. *P. eucalypti* on *E. bicostata* on the other hand shows a very significant rise in apparent photosynthesis 24 days after inoculation. This high apparent photosynthetic rate is maintained for some 7-8 days before a significant rise in respiration rate occurs.

It has already been pointed out that the rate of dark respiration in leaves is affected by the photosynthetic activity during the previous light period. Thus Sempio's (1959) data could be interpreted as illustrating the effect of increased substrate level on respiration. Alternatively it could be interpreted as showing that both apparent photosynthesis and dark respiration rise because the pathogen affects some physiological enzyme system common to both, e.g., an enzyme system similar to that suggested by Scott and Smillie (1963), although obviously not that particular system, because that system would involve a rising respiration with a falling apparent photosynthetic rate. By contrast the apparent photosynthetic
<table>
<thead>
<tr>
<th>Days After Inoculation</th>
<th>Photosynthesis as % of uninfected</th>
<th>Respiration as % of uninfected</th>
<th>Days after Inoculation</th>
<th>Photosynthesis as % of initial rate</th>
<th>Respiration as % of initial rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>2</td>
<td>171.2</td>
<td>119.1</td>
<td>10</td>
<td>102.3</td>
<td>109.1</td>
</tr>
<tr>
<td>4</td>
<td>117.7</td>
<td>131.1</td>
<td>13</td>
<td>97.9</td>
<td>95.5</td>
</tr>
<tr>
<td>6</td>
<td>66.7</td>
<td>178.2</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>138.7</td>
<td>283.2</td>
<td>19</td>
<td>90.5</td>
<td>86.8</td>
</tr>
<tr>
<td>11</td>
<td>123.2</td>
<td>433.7</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>45.7</td>
<td>382.1</td>
<td>24</td>
<td>118.4</td>
<td>115.4</td>
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<td>122.3</td>
<td>114.3</td>
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<td>127.5</td>
<td>116.0</td>
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<td>106.5</td>
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<td>106.5</td>
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<td></td>
<td></td>
<td></td>
<td>39</td>
<td>111.9</td>
<td>153.6</td>
</tr>
</tbody>
</table>
and respiration rate data for P. eucalypti on E. bicostata suggest that there is no initial connection at least, between the rise in apparent photosynthesis and the rise in respiration in infected leaves. This is emphasised still further by the fact that the uninfected leaves on infected plants, which showed a significant rise in photosynthesis at 24 days, do not show a significant rise in respiration above the level at 29 days even at 59 days after the plants were inoculated.

There is no evidence in this host pathogen complex of a sympathetic movement of respiration rate with apparent photosynthesis although there is an overlap of the increases in the two metabolic processes between 32 and 59 days after infection.

By contrast there is good evidence that there is an association between the histological observation of the production of "green" areas on the infected host leaves at 23 days and the rise in apparent photosynthesis at 24 days.

There is also evidence of association between the production of necrotic areas on the leaves and fruiting of the pathogen at 30 days and the rise in respiration of the host-pathogen complex at 29-32 days after inoculation.

These observations suggest that in this host-pathogen complex there is no initial connection between the effect of the pathogen on apparent photosynthesis and respiration of
the host. The data also suggest, in view of the correlation noted earlier between the level of apparent photosynthesis and the rate of respiration in healthy leaves, (Pisek and Tranquillini, 1954) that the extra products of increased photosynthesis in uninfected leaves on infected plants are not available for respiration by those leaves. It is possible that this increased photosynthate is transported to the infected leaves which act as a sink to which excess carbohydrate production by uninfected leaves is channelled. This suggestion is supported by the fact that respiration in these infected leaves is at a maximum when apparent photosynthesis in these leaves is falling and that respiration in these leaves only falls when apparent photosynthesis in them is reduced to some 50% of the initial rate after infection.

The data presented in chapters 7 and 8, indicate that the result of infection is to increase the nett assimilation of the leaves of infected plants. Although the rise in respiration is a greater percentage value of the base respiration rate than the rise in photosynthesis is of the base photosynthetic rate, the nett consequence of the increased metabolism is increased fixation of CO$_2$ (The increase of approximately 20% in the photosynthetic rate represents an apparent fixation of CO$_2$ increase of some 10-13 mm$^3$ CO$_2$ cm$^{-2}$ hr$^{-1}$). The increased respiration rate of approximately 36%
means on increased release of CO₂ of only some 1.6 mm³ CO₂ cm⁻² hr⁻¹. On this basis it might be argued that the early disease is potentially beneficial to the growth of the plant. The very great inherent capacity of the genus eucalyptus to replace its crown following damage (Jacobs, 1955), would again suggest that the reduced life of leaves as a result of infection by a leaf pathogen may be of little significance. If these two factors are combined, then it might be argued that leaf infections by this pathogen are likely to be more beneficial than damaging to the growth of the hosts.

Unfortunately no experiments were conducted to compare the dry weight yield of infected and uninfected plants of E. bicostata. Obviously such an experiment should preferably be carried out using genetically uniform material and under environmental conditions which ensure that reductions or increases in yield in the infected plants are the result of inoculation and not of chance infection.

It is also obvious that experiments to trace the translocation pattern in infected and uninfected leaves are desirable. It has been suggested in Chapter 7 that radioactive tracers might be used to detect any movement of material from the infected leaves to the opposite uninfected leaf. It was suggested that if such a movement occurred this might explain the rise in apparent photosynthesis of the uninfected leaf.
i.e. this rise being triggered by some substance coming from the host pathogen reaction in the infected leaf. Similarly an experiment in which radio active material was fed to the uninfected leaf, particularly during the period of accelerated apparent photosynthesis, might indicate whether the increased photosynthate moves out of the uninfected leaf to be respired in the infected leaf, or is transported elsewhere. A series of such experiments using tracers should show the pathways of translocation and metabolic use in both infected and uninfected leaves on infected plants. An investigation of the biochemistry of the host parasite situation is also desirable. The relatively small rise in respiration of this host pathogen complex by comparison with the host obligate parasite combination, may indicate that the pathways of respiration in the two situations are not the same. An assessment of the $\text{C}_6:\text{C}_1$ ratio in infected leaves and uninfected leaves on infected and uninfected plants would contribute greatly to an understanding of the probable biochemical pathways involved. Once these pathways were known a more intensive biochemical investigation of the processes concerned in these pathways would be rewarding. The observation that in this host pathogen complex the rise in apparent photosynthesis is separated in time from the rise in respiration should make this an attractive situation with which to
work by comparison with powdery mildew on wheat or barley where the increased anabolic and catabolic processes show considerable overlap.

It has been pointed out that a study of the physiology of host-pathogen relations has significance in a consideration of the evolution of the parasitic habit (White, 1957). It has been argued that the efficiency of a parasite may be gauged by its ability to live on the food reserves of the host without unfavourably affecting the host's physiology, i.e., without being severely pathogenic (Horsfall and Dimond, 1960). The obligate parasites on resistant hosts are on this criterion not advanced parasites. On susceptible hosts the period of commensalism with these parasites is much longer, sufficiently long for the parasite to reproduce and commence a new life cycle. The period of commensalism is nevertheless relatively short. This is a disadvantage to the parasite; it provides a problem of carry over of inoculum in an unfavourable environmental period or when there are no satisfactory hosts for the parasite to infect. Adaptations in such obligate parasites by way of production of large numbers of spores to exploit fully the microenvironment of the area and the carry over of the parasite on alternative hosts, are possibly significant in overcoming the disadvantage of short life cycles.

Parasitism by an organism provides an ecological niche
for escape from the intense competition for the colonisation of dead substrates. It gives advantages of time and position to the organism capable of colonising living as distinct from dead substrates (Garrett, 1956). An organism capable of saprophytic existence following parasitic colonisation has a means of surviving in unfavourable environmental periods or in the absence of its host. If saprophytic survival is combined with a long life cycle, the existence of the organism is not seriously threatened by periods of unfavourable environmental conditions or of the temporary absence of the host.

When these factors are taken into consideration _P. eucalypti_ must be regarded as a weak pathogen, but a highly efficient parasite. Its complete elimination would be very difficult to compass.

8. 5 **Conclusions**

1. The mean maximum respiration rate of leaves of _E. bicostata_ infected with _P. eucalypti_ is 31.3% greater than that of comparable uninfected leaves on the same plants at the same time after inoculation. This difference is significant at _P<.001_.

2. The mean maximum respiration rate of leaves of _E. bicostata_ infected with _P. eucalypti_ is 18.4% greater than that of comparable uninfected leaves on the same plant plus the estimated respiration due to the fungus. This
3. From 1 and 2 above it may be concluded that the fungus is contributing some 17.9\% while the infected host cells are contributing some 18.1\% of the increased respiration of infected leaves.

Results and Discussion

Histological and physiological evidence presented indicates that there is a period of at least fifteen days of eusymbiotic (sensu Geumann, 1948) association between

P. eucalypti and L. bicostata following artificial inoculation.

The fungus can be cultured readily on dead substrates. These observations are consistent with classifying the organism as a facultative biotroph (Muller, 1959). Phytophthora infestans and Ustilago sp., in combination with susceptible host varieties, would be classified in the same group (Muller, 1959). There are certain features of the present host pathogen association inconsistent with the behaviour of obligate or facultative biotrophs on susceptible host species.

In certain, if not all, obligate or facultative biotroph and eusymbiotic combinations the fungi concerned commence sporulation prior to host cell collapse (Semple, 1959; White, 1957).

In the case of the combination of Venturia inaequalis and Pyrus malus classified by Muller (1959) as a transitional type between a facultative biotroph and necrotroph association with a promoting the germination of vacant spaces, this latter...
CHAPTER IX

Summary of Results and Discussion

Histological and physiological evidence presented indicates that there is a period of at least fifteen days of eusymbiotic (sensu Gaumann, 1948) association between P. eucalypti and E. bicostata following artificial inoculation. The fungus can be cultured readily on dead substrates. These observations are consistent with classifying the organism as a facultative biotroph (Muller, 1959). Phytophthora infestans and Ustilago sp., in combination with susceptible host varieties, would be classified in the same group (Muller, 1959). There are certain features of the present host-pathogen association inconsistent with the behaviour of obligate or facultative biotrophs on susceptible host species.

In general it has been observed that the inoculum potential of a single biotrophic spore is high (White, 1957).

In certain, if not all, obligate or facultative biotroph eusymbiotic combinations the fungi concerned commence sporulation prior to host cell collapse (Sempio, 1959; White, 1957). In the case of the combination of Venturia inequalis and Pyrus malus classified by Muller (1959) as a transitional type between a facultative biotroph and necrotroph association with a promoting the germination of washed spores. This latter
susceptible host, the pathogen produces spores before the final collapse of the host cells (Nussbaum and Keitt, 1938). At twenty-six days after artificial inoculation the leaves of *E. bicostata* showed extensive chlorotic spots indicative of host mesophyll cell collapse. Sugar to starch conversion had ceased in these areas at least three days previously. Sporulation of the pathogen did not commence until thirty days after inoculation.

A specialised relationship with the host cells is characteristic of many biotrophic fungi (White, 1957). In the cases of rusts, powdery mildews and downy mildews, this specialisation is in the form of haustorial production. With the Archimycete fungi the development of the pathogen is exclusively intracellular. *P. eucalypti* did not show such specialised host cell relationships in any combination examined. Environmental factors variables to behaviour in the

In general it has been observed that the inoculum potential of a single biotroph spore is high (White, 1957) and that the germination of such spores is not increased by the presence of external substrates (Gottlieb, 1964). No spore dose infection curve was produced for the organism but there is considerable evidence that external substrates on the leaves of susceptible hosts are very significant in promoting the germination of washed spores. This latter
observation is however consistent with the observations of other workers on the significance of external substrates in the germination of washed spores of a biotroph (Shepherd and Mandryk, 1963).

In the combination of Septoria passerini and Hordeum sativum (Green and Dickson, 1957) Muller (1959) sees no evidence of even a temporary eusymbiotic relationship. There do not appear to be any other studies of organisms which could be regarded as closely related taxonomically to P. eucalypti.

It seems reasonable to consider the host-pathogen combination as characteristic of the parasitism of a biotroph. In view of the other evidence it is probably more consistent to classify the organism as a transitional type (sensu Muller, 1959).

The difficulties in reasoning from experiments employing a single environmental factor variables to behaviour in the in toto environment have been emphasised for both higher plants and fungi (Lang, 1963; Cochrane, 1958). These difficulties are even more pronounced when the desired environment for study is a host-pathogen complex. Consequently some of the interpretations which follow are more in the nature of fields for possible further analysis than firm conclusions drawn from experimental results.

The period between spore production and the establishment
of a new infection is probably the most critical in the life
cycle of a plant parasitic fungus. A leaf parasitic organism
may purchase time in this critical period by two very obvious
adaptations. Firstly by the spore retaining a high degree of
viability under unfavourable environmental conditions and
secondly by extensive colonisation of a leaf from a single
infection. This second type of behaviour ensures a steady
production of spores from infected material over a considerable
period of time. *P. eucalypti* has neither of these adaptations
and this could be important in "over wintering" (using this
term in a broad sense to indicate periods of unfavourable
environmental conditions). The organism however has certain
characteristics which may be significant in this apparent
difficulty. The spore horn, extruded from a single pycnidium,
because of successive spore production from annellophores,
consists of spores of different ages. The distribution of
such spores, probably by rain spash, is likely to ensure
that at least some droplets contain viable spores to commence
a new life cycle on a susceptible host. It should be noted
in this regard that in the experiment on the relationship
between spore concentration and germination no modification
was introduced to assess the relative significance of viable
and dead spores in the promotion of the germination of viable
spores. It would be very desirable to perform such a test.
The limitation of the parasitic spread of fungal pathogens within leaves is normally considered from the viewpoint of the resistance of the host. However, this limitation may not be without its significance in the ecology of *P. eucalypti*. The necrosis of considerable areas of leaf tissue, e.g., 25 to 30 of total leaf area, is frequently followed by leaf shedding. The shedding of infected leaves in the case of this organism, in eucalypt high forest, would physically remove the organism from the susceptible host tissue in the tree crown. In closed eucalypt stands such removal would most likely break the life cycle since regeneration of tree seedlings under such conditions is very limited.

The proportion of spore germination by host leaves and by the extracts of these leaves has obvious ecological significance for an organism with a long period for germination and a comparatively low viability under normal, e.g., laboratory, conditions. This applies particularly to the observation on the reduced period for germination commencement. The increased total germination of spores on host leaf tissue possibly has considerable significance in the parasitism of hydrophobic type host leaves or leaves held at an angle to the horizontal.

It has been suggested that *E. bicostata* is probably a coloniser of open spaces (Cameron, 1964). The presence of glaucous wax on the juvenile foliage of the species may be
significant in its capacity for such colonisation. These glaucous leaves show a high level of reflectance and absorption and a poor transmission of monochromatic light (Cameron, 1964). The higher germination of the spores of P. eucalypti in darkness than in intermittent high intensity light, is consistent with the colonisation from the underside of glaucous leaves held in a horizontal position. Under nursery conditions the period of seed germination and seedling establishment is critical in the life cycle of the host. Glaucousness and leaf angle both appear to be important factors in the degree of infection of juvenile leaves by the pathogen. The juvenile leaves of E. saligna and E. grandis, both very susceptible hosts, are non-glaucous and are held horizontally. Even the mature leaves of these trees droop rather less than is common with many eucalypts. It should be possible, therefore, to produce more resistant hybrids of these species by crossing with those species which have glaucous or more drooping juvenile leaves. Nevertheless, such a programme should be undertaken with care, since glaucous leaves may be very susceptible to certain other potential eucalypt leaf parasites which are dry air spore e.g., powdery mildew. McNew (1960) has sounded a general warning, particularly applicable to forests, of the dangers of plant breeding against specific parasites.

The in vivo significance of temperature effects on spore
germination is likely to be expressed through the level of humidity associated with high temperatures. The humidity level in summer, possibly limits the spread of the organism onto undoubtedly susceptible eucalypt species west of the Dividing Range.

In the field, species of the subgenus Macranthera (Pryor, 1959) are susceptible to P. eucalypti leaf spot. Under nursery and artificial inoculation conditions the spectrum of susceptibility in the eucalypts is extended to include all species inoculated. These observations are consistent with postulating the inoculum potential as the major factor governing host infection. It seems likely that the susceptibility under artificial inoculation conditions of apparently field resistant eucalypt species may be a further instance of "Wilson's disease of broad beans" phenomenon (Garrett, 1960). This hypothesis does not explain why a higher inoculum potential (used in a broad sense) is required to establish a progressive infection on for example, E. pilularis, than on E. saligna. These two species occur in admixture on the North Coast of N.S.W., where the pathogen appears to be very active on the Macranthercus species but has not been recorded on E. pilularis. This particular instance of host resistance and susceptibility is worthy of investigation since, under conditions of identical behaviour can be interpreted as adaptations to ensure its high spore load with artificial inoculation, the leaves of perpetuation under unfavourable environmental conditions. It
E. pilularis are much less susceptible than those of E. saligna.

The study of the histology and physiology of the host pathogen combination apart from its inherent interest and its contribution to the field of host pathogen relationships in plants, has provided a pattern of behaviour for a eusymbiotic combination of this organism and a susceptible host. A similar study of the organism in combination with a field resistant host would provide an interesting comparison and might indicate a degree of active resistance dependant on the host reaction to the pathogen.

An extension of the investigation of the Axeny type factors significant in resistance to infection is also justified. Such factors may be of considerable importance from an epidemiological view point. They may largely explain the incidence of the disease in the field and also the distribution of the disease on particular parts of the same plant.

This study indicates that P. eucalypti is a parasite of considerable efficiency and specialisation with a high degree of adaptation to its habitat on eucalypt leaves. Certain taxonomic characteristics of the organism and its parasitic behaviour can be interpreted as adaptations to ensure its perpetuation under unfavourable environmental conditions. It
is likely to continue as a biotic factor in the environment of certain eucalypt forest. Because of its limited pathogenicity and relatively long life cycle, it is unlikely to cause significant epiphytotics even under very favourable environmental conditions (Van der Plank, 1960).

Its major growth impact in eucalypt forest is likely to be on dense, uniform seedling regeneration of susceptible species under high humidity conditions. Under such conditions it may reduce the stocking below the optimum desirable for future stem selection at a more mature age, and thus have a direct bearing on sylvicultural practice.
Introduction

The details of experimental methods, results and discussion include the investigation of the promotion of the germination of the spores of E. bicostata by the exudate of E. bicostata. Experiments 14 - 36 dealt with the preliminary investigation on this promotion of spore germination. They are concerned with the more precise definition of the project and the elimination of possible factors causing major promotion of spore germination.

Experiments 11a - 36a, given. The procedure followed to isolate and purify a major promoter of spore germination.

Preliminary Investigation

Considerable quantities of plant material are required for a project of this type. The supply of juvenile leaf material from phytotron raised plants of E. bicostata was very limited. Adult type leaves of E. bicostata were readily available and it was hence preferable to use this foliage if evidence was first obtained of the stimulation of spore germination by these of leaves.

Expt. No. 1. Germination of E. eucalypti spores on juvenile and adult leaves of E. bicostata and on glass slides.

Three unharmed adult leaves of various ages were selected, together with 7 juvenile leaves from the 3rd, 4th
Introduction

The details of experimental methods, results and discussion included here are concerned with the investigation of the promotion of the germination of the spores of *P. eucalypti* by the water soluble leaf exudate of *E. bicostata*. Experiments 1a - 10a deal with the preliminary investigation on this promotion of spore germination. They are concerned with the more precise definition of the project and the elimination of possible chimeras of true promotion. Experiments 11a - 36a, give the procedure followed to isolate and purify a major promoter of spore germination.

Preliminary Investigation

Considerable quantities of plant material are required for a project of this type. The supply of juvenile leaf material from phytron raised plants of *E. bicostata* was very limited. Adult type leaves of *E. bicostata* were readily available and it was hence preferable to use this foliage if evidence was first obtained of the stimulation of spore germination by these of leaves.

Expt. No. 1a Germination of *P. eucalypti* spores on juvenile and adult leaves of *E. bicostata* and on glass slides.

Three undamaged adult leaves of various ages were selected, together with 3 juvenile leaves from the 3rd,
TABLE 1a - 1 Germination of spores of *P. eucalypti* on juvenile and adult leaves of *E. bicostata* and on glass slides.

3 replicates of each treatment (in the case of the juvenile leaves replicates 1, 2 and 3 being from 3rd, 4th and 5th node above cotyledons).

Mean number of germ tubes per group of 25 spores

<table>
<thead>
<tr>
<th>Germination</th>
<th>Replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>Adult Leaves</td>
<td>23.5 ± .72</td>
</tr>
<tr>
<td>Juvenile Leaves</td>
<td>23.9 ± .84</td>
</tr>
<tr>
<td>Slides</td>
<td>1.8 ± .02</td>
</tr>
</tbody>
</table>


and 5th node of a 12" high plant from the phytotron. These were set up in individual petri dishes. Three glass slides were placed in other dishes. The set up of these dishes, the inoculation, incubation, clearing, staining and scoring for the number of germ tubes per group of 25 spores (8 replicates per treatment) were identical with the methods described for similar experiments in Chapter 3.

The results are presented in Table 1a-1

Discussion

The differences between the germination levels on E. bicostata juvenile and adult leaves are not significant at P = .05. In addition the qualitative difference evident between spores germinated on E. bicostata and those germinated on slides or various other leaves (Chapter 3) were evident on both adult and juvenile type leaves. It was decided to use the adult leaves for the isolation of the germination promotive factor.

Expt. No. 2a Germination of P. eucalypti spores on glass slides in the presence of E. bicostata leaf tissue.

In this experiment 6 clean glass slides were taken and placed in petri dishes whose bases were covered with wet vermiculite and filter paper as described previously. A large drop of spore suspension concentration 32,000 per ml,
TABLE 2a - 1 Germination of spores of P. eucalypti on slides in the presence of pieces of leaf tissue of E. bicostata

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Leaf tissue present</td>
<td>23.9 ± .81</td>
</tr>
<tr>
<td>Leaf tissue absent</td>
<td>1.9 ± .03</td>
</tr>
</tbody>
</table>

Results:

TABLE 3a - 1 Germination of spores of P. eucalypti in buffered solutions of E. bicostata leaf extract and distilled water. (4 replicates were prepared of each treatment).

<table>
<thead>
<tr>
<th>Germination Medium</th>
<th>Replicate number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Leaf extract</td>
<td>46.4 ± 1.3</td>
</tr>
<tr>
<td>Distilled water</td>
<td>28.3 ± .72</td>
</tr>
</tbody>
</table>
adjusted to pH 4.0 with Potassium hydrogen phthalate buffer was placed on each slide. A 5 cm square piece cut from undamaged adult leaves of E. bicostata was added to each of three of the slides selected at random. The plates were incubated for 24 hours at 25°C in the dark. The slides were removed from the dishes and the slices of leaf tissue taken from the spore drops to which they had been added. The drops were dried on the slides, using a hot air drier. One drop of Lacto-Phenol cotton Blue was placed on the dried germination drop and covered with a coverslip. The germination was assessed as in Expt 1a. The results are presented in Table 2a - 1.

Discussion

These results confirm those of Expt 1a, that pieces of the adult leaves of E. bicostata are active in promoting germination of the spores of P. eucalypti. The germination obtained was quantitatively and qualitatively similar to that on whole leaves of either juvenile or adult foliage. The results obtained also make it highly unlikely that physical contact phenomena are the major factors in the stimulation observed. Very few of the spores would have made physical contact with the leaf pieces but the germination in the drop was high and uniform.

These experiments together with those of Chapter 3
suggest that the water soluble (at least in the presence of the complex mixture of materials coming out of the leaf) factor is the most likely explanation of the observed promotion of spore germination.

Expt 3a Germination of spores of *P. eucalypti* in a water extract of *E. bicostata* leaves

200 gms of adult *E. bicostata* leaves were selected, the midribs and any obviously damaged tissue were cut away. The leaves were cut up into pieces approximately 0.5 cm square and soaked in a beaker of glass distilled water for 70 hours. The exudate was vacuum filtered to remove the leaf material.

A clear light yellow solution was obtained. An experiment was set up to test the germinative potential of this crude extract. The germinations were carried out in glass staining dishes as described for previous in vitro experiments.

4 replicates were prepared of each treatment. The germination solutions were incubated for 48 hours at 25°C in the dark. The results are given in Table 3a - 1.

Discussion

The germination in the crude extract was qualitatively comparable with that which occurred on intact adult and juvenile leaves of *E. bicostata*. The quantitative promotion of spore germination was approximately 70% (Sig at *P* < .01).

The following are some of the possible causes for the
observed a promotion of germination at particular pH levels. It was noted that the promotion observed could have been related to the time at which the germination was assessed, or the particular buffer in which the germinations were conducted. The promotion could also be the result of a physical factor, such as the colloidal nature of some of the materials in the extract.

It is not possible to entirely eliminate some of the above possibilities. Two further experiments were conducted to at least partially eliminate 1 and 2. It is reasonable to expect that germination of spores of *P. eucalypti* in buffered solutions of distilled water and leaf extracts at various pH levels. It is worthy of note that this experiment was conducted as described previously for in vitro experiments in glass dishes. A series of solutions of buffered distilled water and leaf extract were prepared. One series consisted of potassium hydrogen phthalate buffers, a second series of distilled water adjusted to various pH levels with potassium hydrogen phthalate buffers, a third series of crude extract adjusted to pH levels...
with Potassium hydrogen phthalate buffers and finally a
series of crude leaf extract adjusted to various pH levels
with Citric Acid Phosphate buffers. In each dish the germ-
ination medium consisted of .4 ml of either distilled water
or crude leaf extract + .1 ml of appropriate buffer. To
each dish .1 ml of washed spore solution was added. The petri
dishes containing the dishes were incubated at 25°C in the
dark for 38 hours.

Results:

The results are plotted in Fig. 4a - 1 and 4a - 2

Discussion

It is evident from these results that the promotion of
spore germination by the crude extract occurs over a wide
range of pH and in both buffer systems. It is reasonable to
conclude that the promotion is not a pH effect although the
degree of promotion is modified by the pH of the system
and the buffering materials used. It is worthy of note that
the germination in the crude extract falls below that in
distilled water above pH 5 in Potassium hydrogen phthalate
buffer. At this level and above the amount of sodium hydroxide
used in this buffer system is considerable. This depression
does not occur in the Citric Acid phosphate buffer where a
strong base is not employed to obtain the alkaline pH levels.

In the Citric Acid phosphate buffer system, the crude
Germination of spores of *P. eucalypti* in distilled water and *E. bicostata* leaf extract buffered with Citric Acid phosphate buffer.
Mean ontogenetic germination score for 25 Spores

Leaf Extract

Distilled Water

pH
Germination of spores of *P. eucalypti* in distilled water and in *E. bicostata* leaf extract buffered with Potassium Hydrogen phthalate buffers.
leaf extract and the distilled water show a double optimum curve. This confirms the suggestion, made in Chapter 2, that this depression is a pH controlled availability phenomenon.

Expt 5a Time course of germination of spores of \textit{P. eucalypti} in \textit{E. bicostata} leaf extract

Sixteen clean glass staining dishes were taken. 4 ml of distilled Water + .1 ml of Potassium hydrogen phthalate Buffer pH 4.0 were placed in each of eight dishes. 4 ml of crude leaf extract + .1 ml Potassium hydrogen phthalate pH 4.0 were placed in each of the other eight dishes. To each dish was then added .1 ml of washed spore solution. The staining dishes in petri dishes were incubated at 25°c in the dark. The treatments were sampled at intervals over 72 hours.

Results:

The results are presented in Fig. 5a 1

Discussion

These results show that the crude leaf extract has had an effect both on the germination potential of the spore solution and on the speed with which spores have germinated after exposure to the germination medium. The type of stimulation exemplified by the rise in total germination potential is consistent with the view that exogenous materials frequently have their optimum effect on attenuated and old spores (Gottlieb,
FIGURE 5a 1

Time course of germination of spores of *P. eucalypti* in distilled water and *E. bicostata* extract both at pH 4.0.
Mean ontogenetic germination score for 25 spores

Time (hrs)

Leaf Extract

Distilled Water
The phenomenon of acceleration of the germination of spores, which are potentially capable of germination, has received less attention. This suggests that the crude leaf extract has an effect on some early stage of the change over from a low to a high metabolic state. It has been hypothesised that germination is an energy requiring process and the production of this energy, as finally symbolised by germ tube production, can only be achieved when the spore has synthesised the full set of necessary enzymes (Gottlieb, 1964). Any exogenous material which supplied or helped in the synthesis of these enzymes, could be expected to greatly accelerate spore germination.

These two phenomena are not necessarily different. A spore may be regarded as being attenuated or old when it does not germinate in a reasonable time. This apparent over maturity may merely be a form of deep dormancy which can be broken by the supply of some exogenous material or by some physical factor, e.g. heat (Goddard, 1939). The same material or physical factor when applied to a spore in a less deep state of dormancy, might be expected to cause such a spore to form a germ tube sooner than it would in the absence of the particular factor either chemical or physical. This will be most likely if the effect of the material or physical factor is on some relatively early stage of the metabolic process of
germination.

The results of these preliminary experiments appeared to justify further investigation of the crude leaf extract of *E. bicostata* to ascertain some of the properties of the promotive factor of spore germination.

**Expt 6a** To test the heat stability of the spore germination promotive factor of *E. bicostata* leaf extract.

A major practical difficulty which frequently arises in investigations of this type results from the stability of the promotive material. The heat stability of the promotive factor of the crude leaf extract was tested as a criterion of general stability. The following germination media were used:

A. Crude Leaf Extract

B. Crude Leaf Extract 50 cc reduced to 25 cc by boiling on an electric hot-plate.

C. Crude Leaf Extract 50 cc concentrated by boiling to 5 cc and finally dried off at 45°C in an oven overnight.

A brown residue was left in an oily mixture. This residue was restored to 50 cc with distilled water and the oil separated off in a separating funnel. This solution was filtered and the filtrate used.

D. Distilled water.

The experimental procedure was as detailed previously.
TABLE 6a.1  Germination of spores of *P. eucalypti* in *E. bicostata* leaf extract which had been subjected to heat treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Crude Leaf Extract</td>
<td>67.1 ± 2.91</td>
</tr>
<tr>
<td>B. Extract reduced to 1/2 vol. by boiling</td>
<td>63.3 ± 3.08</td>
</tr>
<tr>
<td>C. Dried and redissolved extract</td>
<td>70.8 ± 1.41</td>
</tr>
<tr>
<td>D. Distilled water</td>
<td>21.6 ± 1.08</td>
</tr>
</tbody>
</table>

TABLE 7a.1  Germination of spores of *P. eucalypti* in the dialysed extract of *E. bicostata* leaves.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Ontogenetic Germination Score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28.12 ± 1.81</td>
</tr>
<tr>
<td>B</td>
<td>62.25 ± 2.47</td>
</tr>
<tr>
<td>C</td>
<td>48.87 ± 2.72</td>
</tr>
</tbody>
</table>
Results are presented in Table 6a 1.

These results indicate that the spore germination promoter is heat stable. The difference of means of treatments C and A is not significant at the 5% level. The removal of the oily material and the residue, which did not redissolve on drying, has no significant effect on spore germination.

Expt 7a Test of *E. bicostata* leaf extract to ascertain if promotive material is dialysable

It was desirable to attempt a separation of the promotive material from the bulk of substances in the crude extract, Dialysis, which achieves a separation based on particle size, provides one such means of basic separation. 100 cc of the crude extract was placed in a dialysis bag and allowed to dialyse into 400 cc of distilled water at room temperature. After 48 hours the bag of extract was removed and the 400 cc of distilled water and materials which had diffused out were reduced to 100 cc under reduced pressure. The material which remained in the bag was also reduced to 100 cc. A germination experiment was set up using the following germination media and the same procedures as described previously.

**Treatments**

A. Distilled Water

B. Material which diffused out of the dialysis bag

C. Material which remained in the dialysis bag
The results are presented in Table 7.1.

These results indicate that at least some of the promotive factor has diffused out, i.e., the material is dialysable. Dialysis, apart from Donnan Equilibrium effects, can only be expected to achieve equilibrium in concentration of a particular material inside and outside the bag. Germination promotion by the material left inside the bag is consequently to be expected. Several hypotheses could be advanced to explain the higher promotive effect of the material which diffused out of the bag compared with that remained inside.

a. The crude extract consists of inhibitors and promoters and that dialysis achieves a differential separation of these inhibitory and promotive materials.

b. The crude extract contains one or many promoters which have different promotive abilities at different concentrations and that dialysis has shifted the concentration of the promoter(s) towards the optimum level.

c. For some reason e.g., Donnan Equilibrium Effect, the concentration of promoter in the material which diffused out is greater than that in the material which remained in the bag.

Expt 8a Test of the Heat stability of the promoters of spore germination in the dialysed extract of E. bicostata leaves.
### TABLE 8a 1 Germination of spores of *P. eucalypti* in boiled, dialysed *E. bicostata* leaf extract.

<table>
<thead>
<tr>
<th>Results</th>
<th>Mean Ontogenetic Germination Score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>34.4 ± 0.87</td>
</tr>
<tr>
<td>B</td>
<td>47.9 ± 1.86</td>
</tr>
<tr>
<td>C</td>
<td>44.5 ± 1.24</td>
</tr>
</tbody>
</table>

### TABLE 9a 1 Germination of spores of *P. eucalypti* in dialysed and ashed dialysed extract of *E. bicostata* leaves.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Ontogenetic Germination Score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>32.5 ± 0.37</td>
</tr>
<tr>
<td>B</td>
<td>52.4 ± 0.73</td>
</tr>
<tr>
<td>C</td>
<td>34.6 ± 0.57</td>
</tr>
</tbody>
</table>
It is possible that the crude extract contained a number of promoters, the heat stable ones of which remained in the bag and the heat unstable ones of which diffused out. The heat stability of the material after dialysis was tested. Fifty cc of the dialysed material was reduced to 25 cc by boiling vigorously and a germination test, as described previously, set up using A. Distilled water, B. Dialysed material reduced to half volume by boiling and restored to original volume with distilled water and C. dialysed material unboiled. The results are presented in Table 8a 1.

These results indicate that the promoter(s) is dialysable and heat stable.

Expt 9a To test the possibility that the promotive factor might be an inorganic ion. The promoter(s) is heat stable and a relatively small molecule, (probably under 600 molecular wt).

A. There is a possibility that such a material is an inorganic ion i.e. the promotion observed is a pure inorganic nutrient effect.

B. Twenty five cc of dialysed material were reduced to 2 cc by boiling, brought to dryness in an oven at 45°C and then ashed in an oven at 350°C for 2 hours. The residual material was redissolved in 25 cc of distilled water. A
germination test was then carried out as described previously, using the following germination media.

A. 4 ml Distilled Water
B. 4 ml Dialysed Material
C. 14 ml Dialysed Material ashed redissolved

The results are presented in Table 9a1.

These results suggest that the promotion is not a simple inorganic nutrient effect. A further germination test was set up to substantiate this.

Expt 10a  Test of the germination of spores of *P. eucalypti* in complete nutrient solution.

It was reasoned that if an inorganic nutrient acted as a germination promoter, then germination promotion should be achieved by the addition of a complete nutrient solution. The following treatments were applied in a germination experiment set up and assessed as described previously.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>4 ml Distilled Water +</td>
</tr>
<tr>
<td>B.</td>
<td>3 ml Distilled Water + 1 ml Hoagland's Solution</td>
</tr>
<tr>
<td>C.</td>
<td>3 ml Distilled Water + 1 ml Dialysed extract</td>
</tr>
<tr>
<td>D.</td>
<td>2 ml Distilled Water + 1 ml Dialysed extract + 1 ml Hoagland's solution</td>
</tr>
</tbody>
</table>

The results are presented in Table 10a1.
TABLE 10a 1 Germination of spores of _P. eucalypti_ in Hoaglands nutrient solution and dialysed _E. bicostata_ leaves extract with additional nutrient.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Ontogenetic Germination Score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>61 ± 1.5</td>
</tr>
<tr>
<td>B</td>
<td>66.5 ± 0.98</td>
</tr>
<tr>
<td>C</td>
<td>77.1 ± 1.55</td>
</tr>
<tr>
<td>D</td>
<td>80.5 ± 1.22</td>
</tr>
</tbody>
</table>

TABLE 11a 1 Germination of spores of _P. eucalypti_ in the ether soluble fraction of the dialysed extract.

<table>
<thead>
<tr>
<th>Germination Medium</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>36.12 ± 0.60</td>
</tr>
<tr>
<td>B</td>
<td>39.12 ± 1.09</td>
</tr>
<tr>
<td>C</td>
<td>55.5 ± 1.90</td>
</tr>
</tbody>
</table>
The means of treatments A and B differ significantly at P = .05 level. The difference of the means of treatment D and C are not significant at the .05 level.

**Discussion**

The results of Expts 9a and 10a, make it very unlikely that the whole of the promotion of germination by the extract is due to a simple inorganic nutrient deficiency in distilled water. The addition of Hoagland's solution to distilled water does produce a promotion of spore germination of some 8% (significant at $P < .05$). This is the only evidence in the experiments of significant promotion by inorganic materials. The promotion of germination obtained by the addition of the same volume of extract to distilled water, is over 25%. This difference in the degree of promotion appears to require an explanation other than that due to inorganic nutrient ions.

It seems reasonable to proceed on the assumption that the major promotion is due to a heat stable dialysable organic material.

**Intensive investigation of the factor in E. bicostata leaf extract causing major promotion of spore germination of P. eucalypti.**

The preliminary investigation indicated that the major promotion of spore germination was probably a heat stable dialysable organic material. The following series of
experiments aimed at isolating and purifying the major promotive material present in the leaf extract.

Investigation on the solubility of the promotive factor in ether.

If as indicated the promotive factor is a dialysable organic material this may be partially soluble in organic solvents. The solubility of the factor in ether was tested first since this solvent is more convenient to handle than the more polar less volatile solvents. If the promotive factor could be moved into an organic solvent this would reduce the complexity of the mixture in which the factor occurs.

Expt 11a Germination of spores of *P. eucalypti* in the ether soluble fraction of the dialysed leaf extract.

The following preliminary steps were carried out to prepare the germination media:

A. Distilled water - 50 cc shaken with equal volume of ether the three times in a separating funnel and the ether soluble fractions separated. The three ether soluble fractions were combined and dried off under reduced pressure and 50 cc of distilled water added to the dried flask.

B. 25 cc Dialysed extract was shaken three times with equal volumes of ether and the ether soluble fractions separated.

The ether soluble fractions combined, dried off and
residual material redissolved in 25 cc of distilled water. All the residue did not redissolve in water, this was filtered off.

C. The 25 cc of extract which had been shaken with ether and from which the ether solubles had been mainly removed, were dried off and redissolved in 25 cc of distilled water. The above solutions were then used as media in a germination test set up as described previously.

Results are presented in Table 11a - 1. Student's T test shows that mean of treatment B is significantly different from the mean of treatment A (P < .05). Mean of C is significantly different from the mean of A or B (P < .01).

The results suggest that the promoter is slightly soluble in ether. The higher germination promotion by the extract which has been washed with ether, than by the ether solubles, suggests that the material, as it exists in the extract, is not particularly ether soluble. Alternatively the extract may have a whole series of promoters only some of which are ether soluble. A possible explanation of the first of these alternatives is that the promoter is in the form of a salt which is relatively ether insoluble. The acidification of such a salt might make it more ether soluble by converting it to the corresponding acid (Hydrolysis of the
active material to inactive compounds is the danger of this procedure). In addition the germination promoter may be more soluble in a more polar solvent.

Expt 12a Germination of the spores of *P. eucalypti* in the ether and ethyl acetate soluble fractions of the acidified dialysed leaf extract.

The following procedures were followed to produce the series of germination media for this experiment:

A. Distilled water which had been washed three times with an equal volume of ether and then with ethyl acetate.

The ether soluble and ethyl acetate soluble fractions were combined and evaporated to dryness. The residual material (there was none apparent) was then made up to the original volume in distilled water.

B. The dialysed leaf extract before washing with ether.

C. The dialysed extract was adjusted to pH 2.0 with Hydrochloride acid (concentrated), washed three times with an equal volume of ether, the ether soluble fractions combined and taken to dryness under reduced pressure. The residual material was redissolved in the original volume of distilled water. Some of the ether soluble material did not redissolve, this was filtered off.

D. The extract, which had been washed with ether, was washed with ethyl acetate three times. The ethyl acetate soluble
TABLE 12a 1. Germination of the spores of P. eucalypti in the ether and ethyl acetate soluble fractions of the acidified, dialysed, leaf extract.

<table>
<thead>
<tr>
<th>Germination Medium</th>
<th>Mean ontogenetic germination score for 25 spores</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$43.9 \pm 1.57$</td>
<td>Few germ tubes</td>
</tr>
<tr>
<td>B</td>
<td>$88.1 \pm 1.79$</td>
<td>Numerous long germ tubes</td>
</tr>
<tr>
<td>C</td>
<td>$68.1 \pm 1.59$</td>
<td>Numerous short germ tubes</td>
</tr>
<tr>
<td>D</td>
<td>$71.1 \pm 1.12$</td>
<td>Numerous short germ tubes</td>
</tr>
<tr>
<td>E</td>
<td>$43.1 \pm 0.86$</td>
<td>Few germ tubes</td>
</tr>
<tr>
<td>F</td>
<td>$43.6 \pm 0.62$</td>
<td>Few germ tubes</td>
</tr>
</tbody>
</table>


fractions from the three washings were combined, dried and the residue redissolved in the original volume of distilled water. Such promoters, hence concentrates on the spore material which had not been washed with organic solvents used. This would indicate the destruction by the concentrated acid when the dialysed extract was acidified.

E. The dialysed extract which had been washed with ether and ethyl acetate was taken to dryness under reduced pressure and redissolved in original volume of distilled water.

F. Distilled water which had not been washed with ether or ethyl acetate.

A germination experiment using the above preparations as germination media, was performed as described previously. Results are presented in Table 12a - 1.

Discussion

These results show that the ether and ethyl acetate soluble fractions of the acidified dialysate give a germination promotion of approximately 32% and 40% respectively. The differences between the means of these germinations and those in the distilled water treatments are both significant at P < .01 level. The mean germination in the ethyl acetate soluble fraction is not significantly better at the 5% level than the germination in the ether soluble fraction.

There is a significant quantitative difference between the germination in the dialysed extract and in the ether or ethyl acetate soluble fractions of the extract. There is also a qualitative difference between the spore germinations in the same media. This suggests that there could be promoters
of both germ tube formation and germ tube growth which are not soluble in the organic solvents used. Such promoters, since they would only be soluble in very polar solvents, would be very difficult to isolate and characterise. Attention was hence concentrated on the promotory materials which had dissolved in the organic solvents.

The failure of the extract after washing with organic solvents (treatment E) to show any promotion is probably not due to the complete removal of these by the organic solvents (this would be in conflict with suggestion in the paragraph above) but to their destruction by the concentrated acid when this washed extract was dried.

There is no significant difference in the germination of P. eucalypti spores in treatments A and F. This indicates that the promotion obtained in the organic solvent soluble fractions is not due to impurities in the solvents. In future experiments it was unnecessary to use distilled water which had been washed with organic solvents as the control medium.

The results of the experiments performed so far would indicate that the water extract of E. bicostata leaves contains a heat stable, dialysable, factor which promotes the germination of the spores of P. eucalypti. This factor (or at least portion of it) is soluble in organic solvents. It was decided to make a bulk extraction of E. bicostata leaves to obtain a
Preparation of a bulk water extract of *E. bicostata* leaves.

5 kilograms of fresh, undamaged, mature *E. bicostata* leaves were collected. The midribs were removed from these and they were mixed in a Waring Blender with 5 litres of distilled water. After crushing and mixing the leaf material was soaked in the water for 24 hours. The leaf material was filtered off under vacuum and a brown filtrate was obtained. The 5000 cc of filtrate was then dialysed into 11,000 cc of distilled water for 48 hours. The 11,000 cc of dialysate was then reduced to 5000 cc by evaporating under reduced pressure. In this evaporation a large quantity of yellow powder was obtained. This powder was filtered off to leave a dark brown liquid.

4000 cc of the dialysed, filtered extract was taken and its pH adjusted to 1.5 with concentrated hydrochloric acid. The extract was washed with a series of organic solvents. In each instance 1500 cc, 800 cc and 500 cc of solvent were used in three successive washings.

Before and after washing with each solvent a 50 cc sample of the extract was taken. The pH of this sample was then readjusted carefully to 4.0 with concentrated Sodium Hydroxide. This had to be carried out with great care adding
the base drop by drop and stirring vigorously. If the pH rose above 6.0 a floculent brown precipitate was formed which did not redissolve when acid was added to bring the pH back to 4.0. Each sample was evaporated to 30 cc under reduced pressure to remove traces of the organic solvents. The volume of each sample was restored to 50 cc with distilled water.

After the washing was completed the dialysed extract was left at pH 1.5 for 48 hours before a 50 cc sample was taken and its pH adjusted to 4.0 with concentrated Sodium Hydroxide (When the next series of germinations were carried out it was realised that this was a mistake which should have been avoided by adjusting the sample immediately after washing).

Expt 13a. Germination of the spores of _P. eucalypti_ in the acidified dialysed bulk extract of _E. bicostata_ leaves before and after washing with organic solvents.

An experiment was set up to test the effect of washing with the various solvents on the germination promotion by the dialysate. Samples of the aliquots with pH adjusted were the germination media used.

Treatment

A. Distilled water.
B. Original Dialysate.

C. Dialysate acidified to pH 1.6 and readjusted to pH 4.0 with base.

D. Dialysate acidified to pH 1.6 and washed with Petroleum Ether and adjusted to pH 4.0 with base.

E. Dialysate washed as in D. then washed with Ether and adjusted to pH 4.0 with base.

F. Dialysate washed as in E. then washed with Benzene and adjusted to pH 4.0 with base.

G. Dialysate washed as in F. then washed with Chloroform and adjusted to pH 4.0 with base.

H. Dialysate washed as in G. then washed with Ethyl Acetate.

(The pH of this sample was not adjusted to 4.0 for 48 hrs, because of an oversight.)

The germination dishes were set up, sampled and assessed as described previously.

The results are plotted in the form of a histogram in Figure 13a 1.

Discussion

The basic hypothesis applied to these results was that a significant rise in germination of the spores in the extract, after washing with a particular organic solvent, indicated that inhibitors had passed into that solvent. A fall in the germination of the spores in the extract, after washing with a
Germination of the spores of *P. eucalypti* in samples of the *E. bicostata* leaf extract before and after washing with each of a series of organic solvents of increasing polarity.

**Key to treatments:**

A. distilled water  
B. Original dialysate  
C. Dialysate acidified to pH 1.6 and readjusted to pH 4.0  
D. Acidified dialysate washed with petrol ether  
E. Acidified dialysate washed with petrol ether and ether  
F. Acidified dialysate washed with petrol ether, ether and benzene  
G. Acidified dialysate washed with petrol ether, ether, benzene and chloroform  
H. Acidified dialysate washed with petrol ether, ether, benzene, chloroform and ethyl acetate.
Mean ontogenetic germination score for 25 spores
particular solvent, indicated that promoters had passed into that solvent.

On this hypothesis the major promotive materials have passed into ethyl acetate while inhibitors have passed into the other solvents particularly petroleum ether.

An experiment was set up to test the organic solvent soluble fractions obtained from the above bulk extraction to confirm this tentative conclusion.

Expt 14a Germination of spores of *P. eucalypti* in the organic solvent soluble fractions of the dialysed bulk extract of *E. bicostata* leaves

The organic solvent soluble fractions obtained as described from the bulk extraction, were dried off under reduced pressure at less than 45°C. When dry the residual materials were dissolved in 4000 cc distilled water (i.e., returned to the original concentration in the dialysed leaf extract).

The ether soluble fraction did not all redissolve. The material which did not redissolve was readily soluble in ethanol. A minimum quantity of ethanol was added to dissolve this residue and the material dispersed in 4000 cc distilled water (referred to hereafter as ether soluble fraction B.).

The organic solvent soluble fractions, redissolved in distilled water were used as germination media in this experiment. The germination dishes with media were set up,
<table>
<thead>
<tr>
<th>Germination Media</th>
<th>Mean Ontogenetic Germination score for 25 spores</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>61.0 ± 1.4</td>
<td>Germ tubes short</td>
</tr>
<tr>
<td>Petrol ether soluble fraction</td>
<td>78.1 ± 1.30</td>
<td>Germ tubes short</td>
</tr>
<tr>
<td>Ether soluble fraction</td>
<td>42.3 ± 77</td>
<td>No germ tubes</td>
</tr>
<tr>
<td>Ether soluble fraction A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ether soluble fraction B</td>
<td>68.1 ± 2.06</td>
<td>Germ tubes long</td>
</tr>
<tr>
<td>Benzene soluble fraction</td>
<td>74.1 ± 1.75</td>
<td>Germ tubes long</td>
</tr>
<tr>
<td>Chloroform soluble fraction</td>
<td>70.1 ± 1.49</td>
<td>Germ tubes short</td>
</tr>
<tr>
<td>Ethyl acetate soluble fraction</td>
<td>34.0 ± 76</td>
<td>No germ tubes</td>
</tr>
</tbody>
</table>
incubated, sampled and assessed as described before.

The results are presented in Table 14a - 1.

Discussion

These results are inconsistent with those of Experiment 13a. The petrol ether soluble fraction, assumed to contain mainly inhibitors from Expt 13a has the greatest significant promotive effect while the ethyl acetate soluble fraction, assumed from that experiment to contain bulk of the promoters, has an inhibitory effect.

It was decided to repeat this experiment using the various organic solvent soluble fractions at a series of lower concentrations. It was thought that isolation of the promoters into relatively pure condition could have affected their activity in relation to concentration behaviour.

Expt 15a Germination of spores of P. eucalypti in the organic solvent soluble fractions, of the extract of E. bicostata, at different concentrations.

The organic solvent soluble fractions of the bulk dialysed, water extract of E. bicostata leaves were used at the following concentrations:

1. 1 ml of the particular organic solvent soluble fraction + 3 ml of distilled water
2. 2 ml of the particular organic solvent soluble fraction + 2 ml of distilled water
3. 3 ml of the particular organic solvent soluble fraction + 3 ml of distilled water
TABLE 15a  Germination of the spores of *P. eucalypti* in the organic solvent soluble fractions of *E. bicostata* leaf extract at different concentration levels.

<table>
<thead>
<tr>
<th>Organic Solvent</th>
<th>Mean ontogenetic germination score for 25 spores Relative concentration of the fraction, in distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>54.5 ± 1.50 61.4 ± 2.24 53.9 ± 1.41</td>
</tr>
<tr>
<td>Ether A</td>
<td>59.1 ± 1.71 71.6 ± 2.13 66.1 ± 1.78</td>
</tr>
<tr>
<td>Ether B</td>
<td>47.0 ± 0.81 48.1 ± 1.33 51.8 ± 2.46</td>
</tr>
<tr>
<td>Benzene</td>
<td>55.8 ± 2.67 57.5 ± 1.30 62.0 ± 1.43</td>
</tr>
<tr>
<td>Chloroform</td>
<td>40.9 ± 1.12 50.5 ± 1.84 51.6 ± 1.95</td>
</tr>
<tr>
<td>Ethyl Acetate</td>
<td>73.5 ± 1.54 64.8 ± 0.58 26.8 ± 0.34</td>
</tr>
<tr>
<td>Control (distilled water only)</td>
<td>49.1 ± 1.42</td>
</tr>
</tbody>
</table>
The germination test was carried out as described previously in glass staining dishes. The results are presented in Table 15a 1.

Discussion

These results are more in conformity with what could be expected from Expt 13a. The ethyl acetate soluble fraction shows the greatest germination promotion at the lowest concentration. There are however some inconsistencies with Expt 14a which make the results rather unsatisfactory e.g. the germinations in the petroleum ether soluble and ether soluble fractions A and B. In addition there was the unsatisfactory germination in dialysed extract after washing in all organic solvents in Expt 13a. Some of these anomalous results could have been due to the acidification to pH 1.6 and possible hydrolysis of active materials. In addition there was the difficulty of adjusting the washed extract back to pH 4.0 with strong base without damaging the promotive materials.

Re-extraction, with organic solvents, of water extract of E. bicostata leaves using a lower degree of acidification. 500 cc of the original dialysed bulk water extract of E. bicostata leaves which had not been washed with organic solvents was taken. This was adjusted to pH 4.0 with concentrated Hydrochloric acid and washed as before using
three washings of 500 cc, 300 cc and 100 cc of each of petroleum ether, ether and then ethyl acetate. After washing with each solvent, the solvent soluble fraction was dried off under reduced pressure at 45°C and the residual materials taken up in 500 cc of distilled water i.e. to the original volume of dialysate. Before and after each washing with each organic solvent a 10 cc sample of the dialysed material was taken reduced to 5 cc on a Rotary Film evaporator at 45°C under reduced pressure, to evaporate the organic solvents and made up to 10 cc again with distilled water. These solvent soluble fractions and dialysed extract before and after each washing were used as germination media in the next experiment.

Expt 16a Germination of the spores of *P. eucalypti* in the dialysed bulk water extract of *E. bicostata* leaves before and after washing with organic solvents and in the organic solvent soluble fractions of this extract.

In this experiment a series of nine glass staining dishes was taken. .3 ml of distilled water, .1 ml of potassium/H phthalate buffer pH 4.0 and .1 ml of washed spore solution were placed in each dish. .1 ml of the appropriate treatment solution was then added to the dish. The dishes were set up, incubated, sampled and assessed for germination as described previously.
TABLE 16a 1 Germination of the spores of P. eucalypti in the dialysed, bulk water extract of E. bicostata leaves before and after washing with organic solvent soluble fractions of this extract.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Ontogenetic Germination Score for 25 spores</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>60.6 ± 1.35</td>
<td>Germ tubes short</td>
</tr>
<tr>
<td>Dialysed extract, E. bicostata leaves</td>
<td>73.1 ± 0.98</td>
<td>Germ tubes short and numerous</td>
</tr>
<tr>
<td>Dialysed extract adjusted to pH 4.0</td>
<td>74.3 ± 1.37</td>
<td>Germ tubes short and numerous</td>
</tr>
<tr>
<td>Dialysed extract after washing with petroleum ether</td>
<td>74.0 ± 0.50</td>
<td>Germ tubes slightly longer and numerous</td>
</tr>
<tr>
<td>Petroleum ether soluble fraction of acidified dialysed extract</td>
<td>40.8 ± 0.74</td>
<td>Germ tubes very short</td>
</tr>
<tr>
<td>Dialysed extract after washing with petroleum ether and ether</td>
<td>77.0 ± 1.47</td>
<td>Germ tubes very long</td>
</tr>
<tr>
<td>Ether soluble fraction of acidified dialysed extract</td>
<td>73.3 ± 1.01</td>
<td>Germ tubes short and numerous</td>
</tr>
<tr>
<td>Dialysed extract after washing with petroleum ether, ether and ethyl acetate</td>
<td>83.8 ± 1.91</td>
<td>Germ tubes very long and numerous</td>
</tr>
<tr>
<td>Ethyl acetate soluble fraction of dialysed extract</td>
<td>73.7 ± 0.81</td>
<td>Germ tubes short and numerous</td>
</tr>
</tbody>
</table>
The results are presented in Table 16a.1.

Discussion

The results of this experiment were generally more consistent than those of experiments 13a and 14a. The petroleum ether soluble fraction of the extract was inhibitory to germination, this was inconsistent with the absence of a rise in the germination in the leaf extract after washing with this solvent. It appeared that washing with petroleum ether and ether removed an inhibitor of germ tube elongation from the dialysed leaf extract. The promotion of germination of the spores by the ether and ethyl acetate soluble fractions of the extract was consistent with preliminary experiments using cut up leaves.

The dialysed extract after washing with all the organic solvents showed a higher degree of germination promotion than did the original extract, or any of the organic solvent soluble fractions of this extract. One of two possibilities could explain this.

1. The dialysed extract consists of a complex of germination promoters and inhibitors. The germination level in the extract is the result of the balance of these promotion and inhibition effects. Washing with the organic solvents removes some promoters but more inhibitors so that the balance in the washed extract favours increased promotion.
2. The alternative explanation is similar but that the major promoter of germination is not soluble in the organic solvents used i.e. the nett effect of washing with organic solvents has been to remove some promoters, some inhibitors but to leave the major promoter behind. A spatial separation of the constituent materials of the original extract and of the organic solvent soluble fractions of this would help to distinguish between these possible explanations.

Paper Chromatography provides a means of such separation. The effectiveness of the separation of the constituent materials by this technique is affected by the complexity of the chromatographed solution i.e. a clearer separation of constituents, at characteristic R.F. (Relative Front Values) is achieved from less complex mixtures of constituents. It was decided to carry out some initial paper chromatography using the ethyl acetate soluble fraction of the dialysed extract. The above experiment indicated that in this fraction the hypothesised balance of inhibitors and promoters favoured promotion. This fraction of the extract probably would be less complex in its constituents than the original extract and hence could be expected to give a more definite separation of the active materials affecting spore germination.

Separation of the constituents of the ethyl acetate soluble fraction of the water extract by paper chromatography.
Strips of Whatman No. 1 Filter paper were cut approx. 2" wide by 7" long. The strips were slightly tapered on the basal end. A pencil line was ruled across the strip approx. 1½" from the basal end. The ethyl acetate soluble fraction of the dialysed extract was spotted in the middle of the base line using a capillary tube, a hair dryer and observing the usual precautions (Block, Durrum & Zweig, 1958). Three chromatograms were prepared in this way. The developing solutions were placed in glass measuring cylinders - approx. ½" of solution in each cylinder. The tops of the cylinders were covered with ground glass tops and the atmosphere in the cylinder allowed 1 hour to equilibrate to the solvent. The solvents used were: Two cylinders Butanol: Acetic Acid: Water 4:1:5 (organic phase after allowing separation for 2 hours) and Acetic Acid: Water 15:85, one cylinder. The spotted papers were introduced into the cylinders so that their bases just touched the level of developing solvent. The developing solvents were then allowed to move up the chromatogram until they came within about ½" of the top. The developed chromatogram was then removed from the cylinder and the solvent front marked with a light pencil line. The papers were allowed to dry. These chromatograms were cut into pieces of equal length, used as media for assessment of germination at different R.F. levels.
Expt 17a  Germination of spores of *P. eucalypti* at various R.F. values on paper chromatograms.

A strip one cm wide, with the original spot in its centre, was drawn from the top to the bottom of each chromatogram. This strip was divided into one cm lengths starting upwards from the base line to the front and taking a 1 cm length below the base line. A number of clean slides, depending on the number of centimetre squares cut from the strip (including one below the base line and one above the front), were prepared in nine cm petri dishes. The base of each petri dish was covered with vermiculite with a filter paper on top. The vermiculite was dampened and a glass slide placed on the paper. Each of the centimetre square pieces from the chromatogram was set in the centre of a glass slide in the separate dishes, and the appropriate R.F. value of the piece marked on the top of the dish.

Two drops of spore solution of *P. eucalypti*, containing approx. 5 x $10^5$ spores/ml, were then placed on each centimetre square. The dishes were incubated for 48 hours at 25°c in the dark. At the conclusion of the incubation period each centimetre piece was dipped into simmering lacto-phenol-cotton blue for ten seconds, then replaced on the appropriate slide. The pieces of paper were mounted in clear lacto phenol and covered with a thin coverslip.
The centimetre squares on the slides were assessed using the high power objective of a microscope with a powerful substage light. The spores were scored using the ontogenetic scoring method. Eight groups of 25 spores were assessed for each R.F. value in each chromatogram.

The germination score for the area below the base line in each chromatogram was taken as the "control" value and the score at the various R.F. values, calculated as a rise (+) or fall (-) in relation to this value.

The rise and fall in germination score at various R.F. values in the three chromatograms were plotted in Fig. 17a 1.

Discussion

The graphs of these results show that there was germination promotion at R.F. values of 0.25 and 0.55 which was reproducible when separate chromatograms were developed in Butanol: Acetic Acid: Water (4: 1: 5). The graphs in addition show the presence of reproducible inhibitors of germination. The plot of the chromatogram developed in Acetic Acid: Water (15: 85) also shows the presence of inhibitors and promoters in the chromatographed material.

This experiment was by way of a leader test to see if the promotion factor could be moved as an entity and if consequently it was possible to use paper chromatography as a means of separating the promoter. No attempt was made to
FIGURE 17a 1

Graphs showing the rise (+) and fall (-) in germination of the spores of *P. eucalypti* on various relative front values on three chromatograms of the ethyl acetate soluble fraction of the dialysed extract of *E. bicostata* leaves.

Chromatogram No. 1 developed in Butanol: Acetic Acid: Water (4: 1: 5)

Chromatogram No. 2 developed in Butanol: Acetic Acid: Water (4: 1: 5)

Chromatogram No. 3 developed in Acetic Acid Water (15: 85)
accurately control the amount of the ethyl acetate soluble fraction of dialysed *E. bicostata* leaf extract spotted on the baseline. Consequently it is to be expected that the chromatograms would not be comparable in all respects.

2. The experiment indicated that the promoters present in the ethyl acetate soluble fraction could be separated as distinct entities by paper chromatography. The experiment also supported the suggestion that the promotion is probably due at least partly to an organic material. It is unlikely that an inorganic nutrient would be moved to an R.F. of 0.55 in Butanol: Acetic Acid: Water (4:1:5).

Comparative paper chromatography of the original water extract and several organic solvent soluble fractions of this extract. It was desirable to establish that, in the ethyl acetate soluble fraction, the original extract and the dialysed extract after washing with organic solvents were chromatographed, there was reasonable comparability between the R.F. values of the promoters in all three materials. This would eliminate the possibility that the promoting areas shown up in Expt. 17a resulted from impurities introduced in the washing with organic solvents. Such a comparison would also indicate which of the two alternative explanations advanced for the high degree of promotion by the dialysed extract after washing with organic length of the chromatogram at which the front was marked.
solvents was most likely to be correct (Expt 16a).

For this purpose the following five solutions were chromatographed on Whatman No. 1 Chromatographic paper:

1. Original Extract
2. Original Extract after dialysis
3. Ether Soluble Fraction of dialysed extract (as used in Expt 16a)
4. Ethyl Acetate Soluble Fraction of dialysed extract (as used in Expt 16a)
5. Water soluble residue after washing with organic solvents (as used in Expt 16a).

In this experiment each solution was streaked across the base line marked on a 4" x 10" strip of Whatman's No. 1 Chromatographic Paper. The strips were cut with their long axis orientated as recommended by the manufacturers. n-Butanol saturated with water was used as the developing solvent. This solvent was prepared by shaking 250 cc of n-Butanol with excess deionised water for 10 mins. The mixture was placed in a separating funnel and allowed to stand for 2 hrs. The organic phase was then separated and used as the developing solvent.

The streaked chromatograms were hung in a closed glass battery case whose atmosphere had been given one hour to equilibrate to the solvent. The solvent ascended the chromatogram very slowly, taking some 4 hrs to climb 8" - the usual length of the chromatogram at which the front was marked.
After development the chromatograms were dried in a fume chamber in a moving air current for 24 hours.

**Expt 18a** Bioassay by spore germination of *P. eucalypti*, of R.F. values of promotion and inhibition in chromatographed extracts.

As described in Expt 17a, one cm wide strips were cut from each chromatogram. This strip was cut into 1 cm lengths, placed on slides in petri dishes and seeded with spores. The incubation, staining and assessment were carried out as described in Expt 17a. The ontogenetic germination score for each R.F. value for each chromatogram was then computed. Each score was then calculated as a percentage rise or fall of the ontogenetic germination score for the area below the base line of that chromatogram. These results are illustrated in Figure 18a 1.

**Discussion**

A comparison the graphed results for the different chromatographed solutions shows a reasonable correspondance of areas of promotion considering the complex mixture of substances which make up the solutions. In particular there is good evidence of a promoter in the R.F. region .35 - .5 present to some degree in all the solutions. It is likely that this promoter corresponds with that found in Expt 17a.
Percentage rise and fall in germination at different R.F. values on chromatograms of the following solutions developed in Butanol saturated with water.

Chromatogram No. 1: Original E. bicostata leaf extract

" No. 2: Dialysed E. bicostata leaf extract

" No. 3: Ether soluble fraction of dialysed E. bicostata leaf extract

" No. 4: Ethyl acetate soluble fraction of dialysed E. bicostata leaf extract

" No. 5: Dialysed E. bicostata leaf extract after washing with the series of organic solvents i.e. petrol ether, ether and ethyl acetate.
at R.F. of .55 when the ethyl acetate soluble fraction of the extract was developed in Butanol: Acetic Acid: Water (4: 1: 5). There is also a promoter present in the Ethyl Acetate Soluble Fraction at an R.F. of approximately .03, which is evident in the original extract.

The R.F. value of a particular material in a particular solvent system under identical environmental conditions will be affected by the presence of other substances in mixture with it. Absolute correspondence in R.F. value of promotory and inhibitory areas in the various chromatograms hence could not be expected. In addition it is obvious that all the solutions chromatographed are complex mixtures of inhibitory and promotive materials. The germination level at any R.F. value will be determined by the balance of promotive and inhibitory effects of the materials present in that area in the particular chromatogram. Finally it is possible that particular materials are inhibitory, promotive or neutral depending on their concentrations. It is to be expected that in chromatograms, such as those prepared, concentration of particular materials at particular R.F. values will vary with the extract chromatographed.

When all the above factors are taken into account the agreement between the R.F. values for germination promoters in the different chromatograms appears reasonably satisfactory.
The results for the dialysed leaf extract after washing with organic solvents compare very well, at the same R.F. values, with those for the ether and ethyl acetate soluble fractions. There is no indication in the former chromatograms of the presence of a major promoter of germination which is insoluble in organic solvents fractions. These conclusions justified further investigation of the ethyl acetate soluble fraction of the dialysed *E. bicostata* leaf extract to isolate the active promotive factor.

Comparative paper chromatography on the ethyl acetate soluble fraction of the extract.

Before the chromatogram strips were used for germination tests in the two previous experiments, the strip was scanned with an ultraviolet light source of wavelength ca 300 μm. It was recorded that a blue fluorescent spot corresponded with an R.F. of .25 in chromatograms of the ethyl acetate soluble fraction of the extract developed in Butanol: Acetic Acid: Water and with an R.F. of .03 in a chromatogram of the same material developed in Butanol saturated with water. These R.F. values were areas of spore germination promotion in both chromatograms. It appeared possible that the material causing the blue fluorescence, might be one of the active promotive materials.

The chromatograms were sprayed with Ammoniacal Silver...
Nitrate, and 1% Ferric Chloride in 95% ethanol containing 1% Hydrochloric acid (Block, Durrum and Zweig, 1958). No reaction was obtained in the fluorescent area although the probable presence of phenolic compounds was demonstrated at higher R,F, values by the production of blue black spots with the Ferric Chloride spray. It was decided to test the purification of several chromatograms similar to those prepared for the germination experiments in Expt 17a were run with a variety of solvent developers. Some typical R.F, values of the fluorescent spot, detected in the previous experiments, in these solvents were:

<table>
<thead>
<tr>
<th>Solvent</th>
<th>R.F, Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol: Water 2: 3</td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate: Formic Acid: Water 10: 2: 3</td>
<td></td>
</tr>
<tr>
<td>Ethyl Acetate: Water</td>
<td></td>
</tr>
</tbody>
</table>

Among the materials, identified as occurring in Eucaluptus leaves, which show light blue fluorescence, R.F, values of approximately this level in these solvents and no reaction to Ferric Chloride are Chlorogenic and Caffeic acids (Block, Durrum and Zweig, 1958).

It was decided to run a further series of chromatograms of the ethyl acetate soluble fraction, using purified samples of both these acids as markers. These chromatograms, developed in Acetic Acid: Water (15: 85), Butanol: Acetic Acid: Water
Isopropanol: Water (2: 3) and Ethyl Acetate: Water (30: 70) showed excellent agreement between the R.F. values of purified chlorogenic acid and the fluorescent spot from the ethyl acetate soluble fraction, in all solvent systems. This indicated that the fluorescent spot was probably due to Chlorogenic acid and it was decided to test the purified acid for its effect on spore germination.

**Expt 19a Effect of Chlorogenic acid at various concentrations on the germination of spores of *P. eucalypti*.**

A 25 mgm sample of C grade Chlorogenic acid was obtained from (California Biochemical Corporation) to prepare a 14 x 10⁻³ Molar solution of Chlorogenic acid. This solution was then diluted to the three levels detailed below. An in vitro germination experiment was set up using these concentrations of Chlorogenic acid as the germination media. The dishes were incubated sampled and assessed for germination as described previously. The results are presented in Figure 19a 1.

**Discussion**

The results show that Chlorogenic acid is an active Promoter of the quantitative germination of the spores of *P. eucalypti* at the concentrations used. In the presence of Chlorogenic acid the spores were very swollen and produced numerous germ tube buds by comparison with those in distilled
Germination of the spores of *P. eucalypti* in C grade Chlorogenic acid obtained from *Calbiochem.* at a series of molar concentrations of the acid.
Mean ontogenetic germination score for 25 spores

Chlorogenic acid ex Calbiochem
Concn. X $10^{-2}$ molar

distilled water
TABLE 20a 1 Effect of Chlorogenic acid from *Fluka* and *Calbiochem* on the germination of the spores of *P. eucalypti*. (Both chlorogenic acid treatments were at $7 \times 10^{-3}$ Molar concentration).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid ex <em>Fluka</em></td>
<td>80.0 ± 1.92</td>
</tr>
<tr>
<td>Chlorogenic acid ex <em>Calbiochem</em></td>
<td>84.7 ± 6.1</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>67.4 ± 9.8</td>
</tr>
</tbody>
</table>

TABLE 21a 1 Germination of spores of *P. eucalypti* in the eluate of a 3 M.M. chromatogram of the ethyl acetate soluble fraction of the water extract of *E. bicostata* leaves.

(Eluate used at 2 concentrations).

<table>
<thead>
<tr>
<th>Percentage eluate in germination medium</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 50</td>
<td>58.4 ± 1.36</td>
</tr>
<tr>
<td>B. 25</td>
<td>52.4 ± 2.45</td>
</tr>
<tr>
<td>C. 0</td>
<td>40.6 ± 1.34</td>
</tr>
</tbody>
</table>

Treatment A. represents a germination promotion of approximately 45% (Sig at P < .001) over C, the distilled water control.
water alone. In distilled water the germ tubes tended to be longer. The overall impression is that the Chlorogenic acid promoted germ tube initiation and restricted germ tube elongation.

The results indicate that chlorogenic acid at a concentration of $7 \times 10^{-3}$ Molar shows maximum promotion of the germination of spores of $P. eucalypti$. Expt 20a, Effect of chlorogenic acid from different manufacturers on the germination of spores of $P. eucalypti$.

Expt 20a was repeated using Chlorogenic acid from two sources, Calbiochem and Fluka, to minimise the possibility that the promotion was due to impurities in the Chlorogenic acid sample. The experiment should establish that the promotion, by Chlorogenic acid from Calbiochem shown in Expt 19a, was reproducible.

The results are presented in Table 20a 1.

Discussion

These results confirm those of Expt 19a that, at the concentrations used, Chlorogenic acid is a quantitative promoter
of the germination of the spores of *P. eucalypti*. At a concentration of $7 \times 10^{-3}$ Molar, the promotion is approximately 20% (Sig @ P < .001). The qualitative effect of Chlorogenic acid was the same as described in Expt 19a.

The comparative chromatography carried out is reasonable proof that Chlorogenic acid is one of the constituents of the ethyl acetate soluble fraction of the dialysed leaf extract of *E. bicostata* leaves.

On this evidence it seems probable that under certain conditions, some of the promotion of the spore germination of *P. eucalypti* by the leaf extract of *E. bicostata* may be due to Chlorogenic acid.

In a discussion of plant growth hormones it has been pointed out that Chlorogenic acid, at low concentrations, is a material which, in the presence of indole acetic acid, increases the growth effect of the hormone. At high concentrations it inhibits growth. (Thimann, 1963). It has been suggested that Chlorogenic acid, which is a common constituent of plants, is likely to be an active inhibitor of sporulation and growth of *Venturia inequalis* (Kirkham, 1959). It is likely that Chlorogenic acid behaves in a similar way to many known promoters of plant growth. At high concentrations it is inhibitory (i.e. it is fungicidal in its effect on spore germination), while at particular levels of concentration it
is promotive (i.e. in this case it promotes the production of germ tube buds). The shape of the curve of results obtained in Expt 19a is in general agreement with this hypothesis.

To determine the in vivo significance of Chlorogenic acid in the pathology of _P. eucalypti_ on Eucalyptus leaves, would require a series of carefully controlled experiments. The leaves of _E. bicostata_ were dipped into ether, the solution concentrated by evaporation and this material chromatographed. It was established by comparative chromatography in three solvent systems that chlorogenic acid probably occurs on _E. bicostata_ leaf surfaces, i.e. the spore can possibly come into contact with the acid before germination.

An assessment of acid concentration on the leaf surface would be the next necessary determination. Even this would not allow a completely satisfactory conclusion. The concentration of Chlorogenic acid in the solution bathing the spore surface is likely to be the significant factor in the effect on spore germination. The significance of the localised concentration of antibiotics in the soil has been discussed elsewhere and is a comparable situation (Garrett, 1957).

One final test was carried out with the Chlorogenic acid from the two commercial suppliers. In Expt 20a the promotion by the Chlorogenic acid from Calbiochem. was significantly greater (P < .05) than that by the same acid obtained from
Fluka, although both were presumably at the same molar concentration. Paper chromatograms of the supposedly pure acid from both sources were run in Butanol: Acetic Acid: Water 4: 1: 5, and dried. The fluorescent spots for the acid were marked on the chromatograms and corresponded identically. The Chromatograms were then sprayed with Ferric Chloride. A blue black spot appeared at R.F. 0.75 in the Chromatogram of the supposedly pure acid from Fluka, while no corresponding spot was evident in the Acid chromatogram. This indicates that the Fluka acid contained an impurity (probably a phenol). The actual molar concentration of the Fluka sample in Expt 20a was probably lower than that of the Acid. This difference in molar concentration probably accounts for the significantly lower spore germination in the Fluka acid by comparison with the Acid.

Any further work with Chlorogenic acid should initially look at the stereoisomers of the acid. (These can be readily separated by two way paper chromatography (Hillis E. pers. comm.). Since chlorogenic acid is readily acid hydrolysable into caffeic and caffylquinic acid, the significance of these two constituent acids in relation to spore germination should be investigated.

Chlorogenic acid is a material of fairly general occurrence
in plants (Karrer 1958). It is unlikely to contribute to the host specificity of a fungus whose spores show an adaptation for increased germination in its presence at certain concentrations. It was decided not to pursue the investigation of germination promotion by Chlorogenic acid. The degree of promotion at R.F. 0.35 - 0.5 in Expt 18a appeared to be greater and this promotive factor could be of significance in the host specificity of the pathogen.

Isolation of a promoter of the germination of spores of P. eucalypti occurring at R.F. 0.35 - 0.5 in paper chromatograms of the leaf extract.

To isolate and purify this promoter it was necessary to obtain adequate quantities of the materials from the active R.F. area. No positive reaction was obtained with a number of chromatographic sprays, bio assay by spore germination was the only criterion available for isolation of the material. Whatman's 3 M.M. paper was selected as being capable of yielding larger quantities of chromatographed solutions. The ethyl acetate soluble fraction of the dialysed leaf extract was streaked across the base line marked on a 10" x 4"
Whatman No. 1 chromatographic paper; except on the extreme left hand edge where a drop of pure Chlorogenic acid was placed as a marker. This paper was developed in n-Butanol saturated with water. The Chlorogenic acid could be readily located in ultra violet light after development in the solvent. The germination on pieces of this chromatogram was assessed as previously described and the R.F. of maximum germination promotion was located at .49. The R.F. of the Chlorogenic acid marker was also marked accurately on this paper.

A 10" x 4" Whatman No. 3 M.M. paper was taken, the ethyl acetate soluble fraction streaked across the base line except at the extreme left hand edge where a spot of Chlorogenic acid was placed. This paper was developed in the same solvent, under the same conditions. The R.F. value of the Chlorogenic acid spot after development was determined under ultra violet light. The ratio of the R.F. chlorogenic acid on 3 M.M. paper

\[
\frac{R_{F_3}}{R_{F_1}}
\]

when applied to some twenty 3 M.M. paper chromatograms of the ethyl acetate soluble fraction of the water extract. The calculated active areas of the chromatograms were combined and eluted. The dried eluate was dissolved in 10 ml of distilled water. This was a 3 M.M. paper. This strip was cut up into small pieces, placed in a filter funnel and eluted with Analar ethanol. The ethanol was evaporated under reduced pressure.
and the residue taken up in 3 ml of distilled water.

Expt 21a. Germination of the spores of *P. eucalypti* concentrations, was used in the eluate from the calculated active on experiment. The R.F. value from each 3 M.M. paper chromatogram previously, of the extract.

In this experiment the eluate obtained above, at two concentrations, and distilled water were used as the germination media. The experiment was conducted as described previously. The results are presented in Table 21.a.1.

**Discussion**

These results indicate that it is possible to use the technique of comparative chromatography to calculate the R.F. of the area of active promotion of germination of spores of *P. eucalypti*. The results also show that it is possible to elute the active promote material from the calculated active area.

This technique of comparative paper chromatography was then applied to some twenty 3 M.M. paper chromatograms of the ethyl acetate soluble fraction of the water extract. The calculated active areas of the chromatograms were combined and eluted. The dried eluate was dissolved in 10 ml of solvent distilled water, propanol in distilled water. This was a lead.

Expts 22a. Germination of spores of *P. eucalypti* in the promoting active bulk eluate from twenty 3 M.M. paper this solvent
chromatograms of the extract.

The bulk eluate derived above, at a series of concentrations, was used as the medium in an in vitro germination experiment. The experimental arrangement was as described previously.

The results are presented in Figure 22a1.

Discussion

This experiment has established the presence of a significant promoter of the germination of the spores of *P. eucalypti* in the eluate from the 3 M.M. paper chromatograms at the calculated R.F. value. The next necessary step in the purification of the active material was to move the promoting factor to another R.F. value by the use of a different solvent system.

Paper chromatography of the active eluate using 80% isopropanol as the developing solvent.

The eluate from the Whatman 3 M.M. Chromatograms, used in Expt 22a, was streaked across the base line of a 10" x 4" sheet of Whatman No. 1 Chromatographic paper. This chromatogram was then developed in a closed glass battery case as described previously. In this instance the developing solvent used was 80% isopropanol in distilled water. This was a leader test to determine if it were possible to move the promoting activity to a different R.F. value using this solvent.
FIGURE 22a 1

Germination of spores of \textit{P. eucalyti} in germinating medium, consisting of the calculated active eluate from fraction of the \textit{E. bicostata} leaf extract developed in Butanol saturated with water.

20 x 3 M.M. Paper chromatograms of the ethyl acetate soluble fraction of the \textit{E. bicostata} leaf extract developed in Butanol saturated with water.
Mean ontogenetic germination score for 25 spores

% Concentration of eluate in germination medium
Expt 23a  Germination of the spores of *P. eucalypti* on a chromatogram of the eluate developed in 80% isopropanol.

A 1 cm wide strip of the dried, developed, chromatogram was cut into 1 cm lengths and the pieces of chromatogram placed on clean glass slides in petri dishes sown with spores and incubated as described previously. Germination was calculated as the mean number of germ tubes per group of 25 spores, 8 groups of 25 spores were assessed on each 1 cm square piece of the strip of developed chromatogram.

These results are plotted in Fig. No. 23a 1.

**Discussion**

There is significant promotion of germination at R.F. .47, .89 and .97 considering the germination on the below the baseline strip as "control". The R.F. .89+ values represent a considerable shift of the R.F. values of .35 - .55 for promotive activity obtained in Expts 18a and 22a, where the solvent system used was Butanol saturated with water. This indicates that it is possible to move the promotive activity as an entity and further paper chromatography should purify the eluate. It is also evident from the results that the eluate contains some significant inhibitors of germination. These are located at R.F. .16 - .37 in the present chromatogram.
FIGURE 2a

Mean number of germ tubes per group of 25 spores of *P. eucalypti* in relation to R.F. value on a chromatogram of the eluate of Expt 29 developed in 80% isopropanol/distilled water.

**Discussion**

There is significant promotion of germination at R.F. 0.48 and 0.39 compared to the germination on the paper. For R.F. 0.89 values, germination as a percentage of the R.F. values of 0.25 for a positive control and a negative control of the R.F. values. This indicates that it is possible to move the homologous activity as an entity and further reduces the chromatography showing partition of the eluate. If the eluate from the germination is mixed with the chromatography showing partition of the eluate it decreases the R.F. 0.48, 0.39 in the present chromatogram.
Mean number of germ tubes per group of 25 spores
Preparation of a chromatopile of the eluate developed in 80% isopropanol. The dishes were incubated, sampled and assayed. It was necessary once again to use the heavier Whatman 3 M.M. paper so that a quantity of material could be eluted from a small number of chromatograms developed in 80% isopropanol. Results are plotted in Figure 24a.

Discussion For this purpose 20 sheets of 10" x 4" Whatman 3 M.M. paper were cut and the base line position ruled at the same level on each sheet. The eluate from Expt 22a was streaked across the base line of each paper. The papers were then built into a chromatopile. The chromatopile was built from the papers clamped between two stainless steel plates. The papers were placed between the plates so that their bases would just touch the top of the developing solvent. After development, the chromatopile was taken apart, the positions of the solvent front marked on each paper, and the papers dried individually in an air stream for 24 hours.

Expt 24a Germination of spores of P. eucalypti at various R.F. values in the chromatopile.

A 1 cm wide strip was then cut up from the middle of one of the central papers of the chromatopile. This strip was then cut into 1 cm lengths and each length placed in an individual glass staining dish containing 0.4 ml distilled water, 0.1 ml Potassium hydrogen phthalate buffer pH 4.4 and
.1 ml washed spore suspension. The R•F• value of each piece was marked on the dish. The dishes were incubated, sampled and assessed as previously described. The Mean Ontogenetic Germination Score for each R•F• value was calculated from counts of 8 groups of 25 spores for each dish.

The results are plotted in Figure 24a 1.

Discussion

The results of this Expt show two areas of significant germination promotion, one at R•F• .78 - .85, the other an apparently more important promoter at R•F• .88 - .98. In this investigation it was intended to try to purify and isolate the most significant germination promoter. These R•F• areas were marked and cut from all papers in the chromatopile (a 1 cm strip either side of the mean values being taken). The R•F• areas were separately eluted with Analar ethanol. The eluates were dried under reduced pressure and the residual material in each instance dissolved in 5 ml of distilled water.

Expt 25a Selection of the most significant promoter of germination from the chromatopile. (The eluates of two active areas eluted above at a variety of concentrations, were used as germination media in an in vitro germination experiment.)

The experimental procedure was as described previously.
Germination of the spores of *P. eucalypti* in relation to R.F. values on a sample sheet of the J M.M. chromatopile of the eluate of Expt 22a developed in 80% isopropanol/distilled water.

**FIGURE 24a 1**

Germination of the spores of *P. eucalypti* in relation to concentration of eluates R.F. .81 and .93 from Expt 31 in the germination medium.

**FIGURE 25a 1**
Mean ontogenetic germination score

% Conc. eluate in germination medium

Concn. e uate in germi n ation medium

R.F. Value

Mean ontogenetic germination score

25 spores
The results are plotted in Figure 25a. 

**Discussion**

The results indicate that the eluate from the chromatogram at an R.F. .93, gave a significant promotion (approx. 25%) when the concentration of the eluate in the medium was only 6.7%. The degree of promotion by the eluate from this R.F. area, decreased with increasing concentration of the eluate in the germination medium. By contrast, the eluate from the chromatogram at R.F. .81 only gave a significant promotion (approx. 13%) at the highest concentration of this eluate in the germination medium, i.e., 16.6% concentration.

Further investigation was restricted to the eluate of R.F. .93 from this chromatopile. A curve of promotion activity in relation to concentration of eluate of R.F. level .93 used in Expt 25a. For this purpose, 2 ml of this eluate were taken and subjected to a 10-fold dilution series with distilled water. One way paper chromatograms of the active promoter were used.

**Expt 26a** Germination of spores of *P. eucalyptii* in relation to the concentration of the eluate, (R.F. .93) from the 80% isopropanol chromatopile, in the germination medium was streaked on the base lines of 20 An in vitro germination experiment was set up to prepare
a spore germination in relation to dilution curve for the eluate R.F. .93 from the chromatopile developed in 80% isopropanol. The experimental procedure was as described previously.

The results are plotted in a histogram form in Figure 26a 1.

Discussion

These results confirm those of Expt 25a, that the eluate of R.F. .93 is a significant promoter of spore germination. The degree of promotion at the optimum level is approx. 25% (Sig. at P < .01). The promotion activity in relation to eluate concentration curve shows a definite peak with a sharp fall to significant but not large promotion on either side of this concentration. The eluate is inhibitory but not statistically significantly so, at the highest concentration used.

It is reasonable to attempt further purification of the promoter by using another solvent system and moving the promoter to a different R.F. level on one way paper chromatograms.

Further purification of the eluate by developing a chromatopile in 40% acetone.

The remaining eluate of the R,F. .93 from the chromatopile run in 80% isopropanol, was streaked on the base lines of 20 Whatman 10" x 4", 3 M.M. papers as described previously.
Germination of spores of *P. eucalypti* in relation to the concentration of the eluate of R.F. 93, from the chromatopile developed in 80% isopropanol.

**Key to treatments:**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% concentration of eluate in germination medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>16</td>
</tr>
<tr>
<td>C</td>
<td>8 x 10^{-1}</td>
</tr>
<tr>
<td>D</td>
<td>8 x 10^{-2}</td>
</tr>
<tr>
<td>E</td>
<td>8 x 10^{-3}</td>
</tr>
<tr>
<td>F</td>
<td>8 x 10^{-4}</td>
</tr>
<tr>
<td>G</td>
<td>8 x 10^{-5}</td>
</tr>
<tr>
<td>H</td>
<td>8 x 10^{-6}</td>
</tr>
<tr>
<td>I</td>
<td>8 x 10^{-7}</td>
</tr>
<tr>
<td>J</td>
<td>8 x 10^{-8}</td>
</tr>
<tr>
<td>K</td>
<td>8 x 10^{-9}</td>
</tr>
<tr>
<td>L</td>
<td>8 x 10^{-10}</td>
</tr>
</tbody>
</table>
Mean ontogenetic germination score for 25 spores

Treatment

A B C D E F G H I J K L
These papers were built into a chromatopile which was developed using 40% Acetone in distilled water as the developing solvent.

Expt 27a  Assessment of germination of spores of *P. eucalypti* on a sample strip from the chromatopile developed in 40% acetone.

The strip of developed dried chromatogram, on 3 M.M. paper, was cut into one cm lengths. These were placed in glass staining dishes and the R.F. value marked. To each of the dishes was added .4 ml distilled water, .1 ml Potassium hydrogen phthalate buffer and .1 ml washed spore suspension. The dishes were incubated, sampled and assessed for ontogenetic germination score as described previously.

The results are plotted in Figure 27a 1.

Discussion

The above results show two areas of statistically significant promotion, one at R.F. .52, the other a much larger promotion (some 26%) at R.F. .76. It was decided to concentrate on the active area at R.F. level .76. A strip .5 cm wide on either side of this R.F. value in each chromatogram was removed. These strips were cut up and eluted with Analar ethanol. The ethanol was evaporated and the residual material, 17 mgms, was dissolved in 5 ml of distilled water. It was noted at this stage that the eluted material appeared
FIGURE 27a 1

Germination of spores of *P. eucalypti* in relation to *R* x *P* value on a sample strip from the 3 M.M. chromatopile developed in 40% Acetone/distilled water.

The results are plotted in Figure 27a 1.

Discussion

The results indicate two phases of activity:

1. Significant promotion of one of *R* x *P*.
2. Significant depression (some 50%) of *R* x *P*.

The other a map for test promotion is at 60% level, while a strip for both is at 40% level.

The results were removed, these results were cut up and mixed with acetone.

The overlap was observed to be abnormal and the results were not as expected.
Mean ontogenetic germination score for 25 spores
to be much less soluble in distilled water than had been the case with elutions from earlier chromatograms. The material was readily soluble in ethanol. The material only became solid after exposure to room temperature for approx. 10 mins after drying on the rotary film evaporator at 40°C. The material was dry and smudgy at room temperature and liquid at 35°C.

Expt 28a To determine the optimum concentration for the major promoter located on the chromatopile developed in 40% acetone.

It had been shown in Expt 26a, that the promoter of spore germination showed an optimum concentration for promotion activity. It was decided to carry out a 10-dilution of the R.F. 76 eluate of the 3 M.M. chromatopile developed in 40% Acetone/distilled water to determine the optimum concentration of this new eluate for germination promotion activity. 1 ml of the eluate (17 mgms of dried material dissolved in 5 ml of distilled water) was taken and subjected to a 10-dilution with distilled water. A germination experiment was set up using the various eluate concentrations as germination media. The dishes were incubated and sampled as described previously.

The results are given in Figure 28a 1.
Germination of spores of *P. eucalypti* in relation to the concentration of eluate in the germination medium C. Eluate, from chromatopile run in 40% acetone - 17 mgms of eluted material dissolved in 5 ml of distilled water.

**Key to treatment concentrations**

A. Distilled water

B. Eluate at original concentration i.e. 17 mgms in 5 ml distilled water

C. " " 10^{-1} dilution

D. " " 10^{-2} "

E. " " 10^{-3} "

F. " " 10^{-4} "

G. " " 10^{-5} "

H. " " 10^{-6} "

I. " " 10^{-7} "

J. " " 10^{-8} "

K. " " 10^{-9} "

L. " " 10^{-10} "

FIGURE 28a 1
Mean number of germ tubes per 25 spores

Treatment
Discussion

The overall results of this test were not entirely satisfactory. The eluate showed a significant depression of germination at the highest concentration used, and a definite peak of germination promotion at $10^{-1}$ concentration, with a drop in promotion (nevertheless significant promotion) down to a concentration of $10^{-3}$. At concentrations between $10^{-4}$ and $10^{-8}$ there are apparent areas of significant inhibition, while at concentrations of $10^{-9}$ and $10^{-10}$ the eluate was neutral in its effect on germination. It is possible that some of these apparent anomalies could have been removed by repeating the test and also by using the ontogenetic scoring method which appears to be much more sensitive and consistent. The results established the presence in the eluate of a very active promoter of spore germination (approximately 200% on this basis of scoring). The purification and isolation of this material was the major aim of this investigation, repetition of the experiment seemed unjustified.

Expt 29a Germination of Spores of *P. eucalypti* in a combination of chlorogenic acid and the eluate of chromatopile run in 40% acetone.

In this experiment the concentrations of chlorogenic acid and eluate used were those which had been shown in previous experiments (c.f. Expt 19a and 28a) to be optimum
TABLE 29a 1 Germination of Spores of P. eucalypti in a combination of chlorogenic acid and active eluate of the chromatopile run in 40% acetone.

<table>
<thead>
<tr>
<th>Germination solution</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>37.7 ± .99</td>
</tr>
<tr>
<td>Chlorogenic acid at 7 x 10^-3 molar</td>
<td>46.5 ± .91</td>
</tr>
<tr>
<td>Eluate at 10^-1 dilution</td>
<td>63.3 ± 1.29</td>
</tr>
<tr>
<td>50% chlorogenic acid at 1 x 10^-3 molar and 50% eluate at 10^-1 dilution</td>
<td>69.6 ± 2.12</td>
</tr>
</tbody>
</table>

TABLE 30a 1 Germination of the spores of P. eucalypti in the ether insoluble fraction of the eluate at various concentrations.

<table>
<thead>
<tr>
<th>Germination Solutions</th>
<th>Mean number of germ tubes per group of 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Original Concentration</td>
<td>8.0 ± .65</td>
</tr>
<tr>
<td>B. diluted to 10^-1</td>
<td>11.1 ± .72</td>
</tr>
<tr>
<td>C. &quot; &quot; 10^-2</td>
<td>13.5 ± 1.09</td>
</tr>
<tr>
<td>D. &quot; &quot; 10^-3</td>
<td>12.9 ± .55</td>
</tr>
<tr>
<td>E. &quot; &quot; 10^-4</td>
<td>10.9 ± .87</td>
</tr>
<tr>
<td>F. Distilled water</td>
<td>11.9 ± .47</td>
</tr>
</tbody>
</table>
for germination promotion. The experiment was set up as described previously.

The results are presented in Table 29a.1. were performed in a comparison of each other and the eluate material was stored

Discussion

These results indicate that Chlorogenic acid at an anionic concentration of \( 7 \times 10^{-2} \) M promotes the germination of \( P. \) eucalypti spores by 23.3% (Sig. @ P < .001). The eluate promotes the germination of \( P. \) eucalypti spores by 68.2% (Sig. @ P < .001). The combination of Chlorogenic acid and the eluate, at the concentration used, gives a promotion of 49.6% (Sig. @ P < .001) over Chlorogenic acid, acid alone and a promotion of 9.9% (Sig. @ P < .01) over the eluate used alone. This emphasises the relative significance of these two promoters of spore germination.

The superiority of the combination of the two promoters over each used individually at their optimum level, suggests that the two materials possibly affect different portions of the germination process. This emphasises the physiological complexity of the germination process as has been discussed elsewhere (Gottlieb, 1964).

A comparison of the percentage promotion shown by the eluate at the same concentration in this experiment by Expt 28a is also interesting. In Expt 28a, using the number of germ tubes method of scoring "germination"
the promotion represented nearly 200%. In this experiment using the ontogenetic germination scoring method the promotion represents only some 68%. The two experiments were performed within 48 hrs of each other and the eluate material was stored in a cold room. These results emphasise that the ontogenetic scoring method is conservative in showing up differences between treatments.

Preliminary fractionation of the eluate by thin layer chromatography and washing with ether.

The eluates used in Expts 22a - 29a, had been isolated and purified by paper chromatography in three solvent systems. It is unlikely that the final eluate could be further purified by one way paper chromatography. Two way paper chromatography could be expected to achieve further separation of the constituent materials in the eluate. The location of promoting areas on two way chromatograms, by a bio assay of spore germination, would present considerable mechanical difficulties.

It was decided to attempt a separation of the constituents of the eluate by thin layer chromatography. The standard procedure recommended for the preparation of heat activated, thin layers, of silica gel on glass slides was followed (Stahl Layers on microscope slides were used in initial experiments. A spot of the eluate was placed in the centre of the base line. The slides were developed in biological staining jars, using
75% benzene and 25% acetone as the developing solvent. Several slides were prepared. These were examined under an ultra violet lamp of wavelength ca 300 mp and the following distribution of fluorescent materials noted.

**Base line** - yellow fluorescent material.

**Front** - slight light blue fluorescence.

**R.F.** .2 -.3 - 3 distinct fluorescent spots all light blue. The leading spot of the three was the strongest.

One of the slides was sprayed with concentrated sulphuric acid. A dark spot was produced on the base line and at the leading fluorescent spot. This indicates that the major concentrations of the organic materials on the chromatogram were on the base line and at the leading fluorescent spot. This chromatogram also indicates that the base line material is relatively polar. In the original separation of the *E. bicostata* extract by washing with organic solvents, it was found that the germination promoter moved into ether (See Expt 18a). It was decided to wash the eluate with excess ether. This washing might achieve a separation of the base line material from the other less polar constituents of the eluate.

4 cc of eluate were washed successively with 10 cc, 5 cc and 5 cc of Analar ether. The ether soluble fraction
was separated on each occasion from the water soluble fraction in a separating funnel. The ether soluble fractions were combined, dried off under reduced pressure and the residual materials redissolved in 4 ml of distilled water. The ether insoluble fraction was taken to dryness and redissolved in 4 ml of distilled water.

Expt 30a Germination of spores of *P. eucalypti* in the ether insoluble fraction of the eluate.

In this experiment the ether insoluble fraction of eluate at various concentrations was used as the germination medium.

The results are presented in Table 30a 1.

Discussion

There is no significant promotion of spore germination of *P. eucalypti* by this ether insoluble fraction at any of the concentrations used. The inhibition of germination of treatment A. by comparison with treatment F. is significant @ P < .05. It is reasonable to conclude that the promoting material has moved into the ether, and this was tested in the next experiment.

Expt 31a Germination of spores of *P. eucalypti* in the ether soluble fraction of the eluate.

It has been observed in the discussion of Expt 27a that the material had become very insoluble in distilled
TABLE 3la 1 Germination of the spores of *P.* eucalypti

in the ether soluble fraction of the eluate of
the chromatopile run in 40% acetone at a
series of concentrations.

(The eluate was suspended in distilled water
and also dispersed by ethanol in distilled
water).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Original concn.</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate in distilled water.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.0 ± .5</td>
<td>18.0 ± .04</td>
<td>15.3 ± .67</td>
<td>13.2 ± .89</td>
</tr>
<tr>
<td>Eluate in ethanol in dist. water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.2 ± .89</td>
<td>30.1 ± 1.14</td>
<td>28.1 ± 1.26</td>
<td></td>
</tr>
<tr>
<td>Ethanol in distilled water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.0 ± .65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.4 ± 1.44</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
water with increasing purification. The ether soluble fraction of the eluate remained as a smudge on the surface of the flask when the ether was dried off and distilled water added to dissolve it. The material was very soluble in ethanol.

The distilled water was dried off and the residual solids dissolved in 4 ml Analar ethanol. 1 ml of the Ethanol solution was taken, the Ethanol dried off and 1 ml distilled water added. A 10 dilution series, to $10^{-4}$, was carried out on this sample. The remaining 3 ml of residual material in ethanol were dried off and the material taken up in 25 drops of ethanol (this appeared to be the minimum quantity necessary to dissolve it). This 25 drops of ethanol containing the residual material was then made up to 3 ml with distilled water. A sample of this was taken and a 10 dilution series prepared of this sample to a concentration of $10^{-2}$. A solution of 25 drops of ethanol was also made up to 3 ml with distilled water.

These media were then used in a spore germination test in glass staining dishes. The experimental procedure was as described previously.

The results are presented in Table 31a 1.

Discussion

The ether soluble fraction of the eluate shows considerable promotion when it is dispersed in distilled water with
TABLE 32a 1  Germination of the spores of *P. eucalypti* in the ether soluble fraction of the eluate at various concentrations.

(The "control" treatments were ethanol alone in distilled water at corresponding concentrations).

Mean number of germ tubes per group of 25 spores

<table>
<thead>
<tr>
<th>Concentration of Treatment material (expressed as a 10⁻¹ dilution of original concentration)</th>
<th>Germination Medium</th>
<th>10⁻¹</th>
<th>10⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluate</td>
<td>(A)</td>
<td>23.3 ± 1.0</td>
<td>20.9 ± 1.39</td>
</tr>
<tr>
<td>Ethanol</td>
<td>(C)</td>
<td>15.1 ± 0.76</td>
<td>11.7 ± 0.82</td>
</tr>
</tbody>
</table>
ethanol. In the absence of dispersal by ethanol this fraction has no significant effect on germination. The promotive material is probably insoluble in distilled water in this semi-pure condition. The promotive effects of the above treatments could be due to ethanol at particular concentrations. A further experiment was performed to compare the spore germination in the eluate dispersed by ethanol in distilled water and ethanol alone in distilled water at the same concentration.

Expt 32a Germination of spores of *P. eucalypti* in the ether soluble fraction of the eluate at various concentrations.

In this experiment the eluate, dispersed in distilled water with ethanol, and ethanol at the same concentration in distilled water, were the germination media used. The experimental procedure was as described previously. The results are presented in Table 32a 1.

Discussion

These results establish that the promotion obtained with the eluate treatments of the previous experiment was largely due to the presence of eluted materials soluble in ether and not due to ethanol. In this Expt treatment A shows a promotion of 54.3% (Sig at P<.01) over treatment C and treatment B a promotion of 78.6% (Sig at P<.01) over
Separation of the constituents of the eluate by thin layer chromatography.

It was evident from the preliminary thin layer chromatography carried out, that this technique provided a further means of purifying the eluate from the paper chromatopile. Four thin layer silica gel chromographic plates, were prepared on 10" x 8" glass plate and activated in the usual way. Spots of the ether soluble fraction of the eluate (used in Expt 32a) were placed on the base line of these plates. Each spot was spaced at 1 cm from the adjoining spots. 2 ml (approx.) of the material were spotted on these plates. The plates were developed in a Benzene 75% Acetone 25% solvent in large covered battery tanks. After development and drying the plates were examined under a u.v. light wavelength ca 300 m\(\nu\). The following distinct spots were apparent:

Baseline - weak yellow fluorescence.
R.F. 20 - weak blue fluorescence.
R.F. 25 - weak blue fluorescence.
R.F. 31 - strong blue fluorescence.
Front - weak blue fluorescence.

The boundaries of strips enclosing each of these areas were marked with a needle and the areas of silica gel enclosed
removed from the plate with a small metal spatula. The particular R.F. areas from the 4 plates were combined to give 5 distinct packages of material which chromatographically appeared to be distinct entities. On one plate, portion of the total plate was left intact. This was sprayed with concentrated Sulphuric acid. A dark spot was produced corresponding to the leading fluorescent spot (R.F. .31) and a weak spot on the base line. Otherwise the plate was clear. This indicated that there were significant quantities of organic materials at these two levels.

The silica gel packages were each placed in separate, small, filter funnels plugged with cotton wool. These packages were then eluted with Analar ethanol. Each eluate fraction was dried under reduced pressure, 12 drops of Analar ethanol were added and each fraction was then made up to 2 ml with distilled water. Each fraction was then subjected to a 10 dilution series to give a concentration ranging from original concentration to 10^{-3}.

Expt 33a Germination of the spores of *P. eucalypti* in the eluates of the chromatographic fractions from the thin layer chromatograms.

The eluates of the chromatographic fractions prepared as outlined were used as media in a germination experiment. The experimental procedure was as described previously.
TABLE 33a 1 Germination of the spores of *P. eucalypti* in
the eluates of the fractions separated in the
thin layer chromatograms at a series of
concentrations. (The fractions were disper-
sed in distilled water with ethanol. Ethanol
in distilled water at the same concentrations
was used as "control").

Mean ontogenetic germination score for 25
spores Concentration of treatment material
in media expressed as a 10⁻ dilution of
original concentration.

<table>
<thead>
<tr>
<th>Germination Medium</th>
<th>10⁻¹</th>
<th>10⁻²</th>
<th>10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>*C.F. Baseline</td>
<td>55.3 ± 0.58</td>
<td>54.3 ± 0.64</td>
<td>56.3 ± 0.72</td>
</tr>
<tr>
<td>C.F.R.F. .20</td>
<td>55.6 ± 0.63</td>
<td>54.5 ± 0.59</td>
<td>55.0 ± 0.57</td>
</tr>
<tr>
<td>C.F.R.F. .25</td>
<td>55.3 ± 0.62</td>
<td>56.9 ± 0.72</td>
<td>57.3 ± 0.84</td>
</tr>
<tr>
<td>C.F.R.F. .31</td>
<td>59.9 ± 1.22</td>
<td>60.5 ± 0.96</td>
<td>58.6 ± 0.62</td>
</tr>
<tr>
<td>C.F. Front</td>
<td>54.7 ± 0.53</td>
<td>55.5 ± 0.49</td>
<td>55.5 ± 0.65</td>
</tr>
<tr>
<td>Ethanol in distilled water</td>
<td>59.3 ± 1.19</td>
<td>54.5 ± 0.61</td>
<td>54.8 ± 0.61</td>
</tr>
<tr>
<td>Distilled water</td>
<td>55.6 ± 0.57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* C.F. = Chromatographic Fraction.
Since ethanol was used to disperse these fractions in distilled water, ethanol in distilled water, at the same concentrations, was used as the "control".

The results are presented in Table 33a 1.

Discussion

The only significant germination promoter from the chromatograms in the system, at the concentrations used, was obtained from the eluate of R.F. .31 in the thin layer chromatograms i.e., the leading and most pronounced blue fluorescent spot. This eluate was most active in promoting spore germination at a concentration of \(10^{-2}\) of the original eluate concentration. It is also evident from the results that ethanol alone in distilled water at a concentration of \(10^{-1}\) is a significant promoter of spore germination.

After the extensive chromatographic purification procedure followed, it seems likely that a single spot such as this would represent a single chemical entity. Further confirmation of this assumption would be obtained by moving the particular spot to another R.F. value by a change in the solvent system used. This was carried out in one of the final experiments. Before attempting this it was thought worthwhile to confirm the results of this experiment using a fresh preparation of ethanol solution and a fresh spore solution.
Germination of spores of *P. eucalypti* in the eluate (R.F. .31) from the thin layer chromatogram dispersed in distilled water with ethanol. (The "control" was ethanol in distilled water at the same concentration).

<table>
<thead>
<tr>
<th>Germination medium</th>
<th>Mean Number: Eluate of R.F. .31 at 10^{-2} of original</th>
<th>Ethanol in distilled water at 10^{-2} concentration.</th>
<th>group of 25</th>
<th>18.6 ± .43</th>
<th>5.6 ± .38</th>
<th>Difference of Means is 23.2% (Significant at <em>P</em> &lt; .001)</th>
</tr>
</thead>
</table>
Expt 34a. Repeat portion of Expt 40 to illustrate reproducibility of results.

This experiment was carried out in glass staining dishes as described in Expt 33a. A new ethanol solution prepared as described previously and diluted to \(10^{-2}\), and a fresh spore solution were used in the experiment. The experimental procedure was as described previously.

The results are presented in Table 34a 1.

**Discussion**

This experiment confirmed the results of Expt 40, that at a \(10^{-2}\) concentration of the original eluate concentration, the eluate of R.F. .31 from the thin layer chromatograms was highly promotive of the germination of the spores of *P. eucalypti*. In this experiment the promotion was 232\% (Sig @ \(P < .001\)) of the control germination in distilled water with ethanol at the same concentration.

Separation of the constituents of the 40\% acetone chromatopile eluate by thin layer chromatography in 65\% benzene, 35\% acetone.

The remainder of the ether soluble fraction of the eluate from the paper chromatopile developed in 40\% acetone/distilled water, was spotted on 6 silica gel activated thin layer chromatographic plates, as described previously. The developing solvent used was 65\% Benzene and 35\% Acetone.
TABLE 35a   1 Germination of the spores of *P. eucalypti* in the eluates of fractions from the thin layer chromatograms at various concentrations.
(The fractions were dispersed in distilled water with ethanol.) Ethanol in distilled water at the same range of concentrations was used as the control.

<table>
<thead>
<tr>
<th>Concentration of treatment material in media (expressed as a 10⁻ dilation of original concentration.)</th>
<th>Mean number of germ tubes per group of 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C.F.</em> R.F. 0.41</td>
<td>17.4⁺ ± 0.99</td>
</tr>
<tr>
<td><em>C.F.</em> R.F. 0.29</td>
<td>16.6⁺ ± 1.60</td>
</tr>
<tr>
<td><em>C.F.</em> R.F. 0.14</td>
<td>8.2⁺ ± 2.02</td>
</tr>
<tr>
<td>Ethanol in distilled water</td>
<td>18.4⁺ ± 1.54</td>
</tr>
<tr>
<td>Distilled water</td>
<td>9.3⁺ ± 0.74</td>
</tr>
</tbody>
</table>

*C.F.* = Chromatographic Fraction
This solvent gave an even better separation of the constituents recorded in the 75% benzene 25% acetone solvent.

Baseline - weak yellow fluorescence.
R.F. .29 - weak blue fluorescence.
R.F. .41 - strong blue fluorescence.
Front - weak blue fluorescence.

As described previously, these areas were separated, eluted, dispersed with ethanol in distilled water and subjected to a ten dilution series to a concentration of $10^{-3}$ of the original eluate.

Expt 35a Germination of spores of *P. eucalypti* in eluates of fractions from thin layer chromatograms.

The eluted fractions were used in a germination test in staining dishes following the usual procedures. In this experiment no test was carried out with the baseline or front materials since it was apparent from previous experiments that those areas were not promotory.

The results are presented in Table 35a 1.

**Discussion**

This experiment shows essentially the same features as Expt 38a. The only chromatographic fraction showing significant promotion, by comparison with its ethanol control, is that from the strong leading blue fluorescent spot. The
TABLE 36a 1 - Germination of spores of *P. eucalypti* in the eluate of R, F, *41* from the thin layer chromatogram at a series of concentrations. (Ethanol in distilled water at the same concentrations was the "control").

<table>
<thead>
<tr>
<th>Concentration of treatment solution in the medium</th>
<th>Germination Medium</th>
<th>Chromatographic Fraction R, F, <em>41</em></th>
<th>Ethanol in distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-4</td>
<td>14.66 ± 1.03</td>
<td>9.58 ± 0.59</td>
<td></td>
</tr>
<tr>
<td>10^-5</td>
<td>13.59 ± 0.90</td>
<td>8.42 ± 0.55</td>
<td></td>
</tr>
<tr>
<td>10^-6</td>
<td>13.27 ± 0.80</td>
<td>7.41 ± 0.80</td>
<td></td>
</tr>
</tbody>
</table>

Mean number of germ tubes per group of 25 spores.
promotion - almost 100% at concentration $10^{-1}$ - due to ethanol in distilled water is also emphasised. In these results significant promotion by the eluate in relation to its ethanol control occurs only at the lowest concentration tested. Even in this case the degree of promotion is low when compared with that obtained in Expt 33a. In the preparation of these chromatograms and elution from them there is no satisfactory control of concentration of materials in the elute in different experiments. It was decided as a final experiment in this series to use the eluate of the chromatographic fractions R.F. 41 at lower concentrations and compare this with ethanol in distilled water at the same concentrations.

Expt 36a Germination of spores of *P. eucalypti* in the eluate of R.F. 41 from thin layer chromatogram at lower concentrations.

Chromatographic Fraction R.F. 41 from Expt 35a, was further diluted in a 10 series dilution to $10^{-6}$. Control ethanol was diluted on the same concentration basis. A germination test, following the usual procedures, was carried out using these germination media.

The results are presented in Table 36a.1.

**Discussion**

The results of this experiment confirm those of Expts 33a - 35a, that the leading strong blue fluorescent spot on
the thin layer chromatogram of the ether soluble fraction of
the eluate of the paper chromatopile run in 40% acetone/distilled water, is an active promoter of the germination of the spores of P. eucalypti. In this experiment the greatest promotion of germination relative to the ethanol control, was at a concentration of $10^{-6}$ of the original eluate concentration, a promotion of some 80% (Sig @ $P < .001$). As mentioned earlier, in these chromatographic experiments there has been no control of the concentration of material in the eluates from the chromatograms. The contradictions between the optimum concentration of the eluate in the medium for germination promotion, are probably more apparent than real.

In the final series of experiments on the eluates of the thin layer chromatograms the same spot at two different R.F. values in different solvent systems has shown the major significant promoting activity. It is reasonable to assume that this material is a major spore germination promoter present in the crude eluate from the paper chromatograms. In view of the purification of the crude leaf extract achieved by dialysis, organic solvent fractionation, paper chromatography and thin layer chromatography, the eluate of this particular spot can be regarded as at least chromatographically pure and possibly it represents a single chemical entity.
The identification of the material responsible for the particular fluorescent spot is a pure chemical problem. A major difficulty throughout this study was the failure to get any positive reaction with the usual chromatographic sprays. At the conclusion of the above experiments no quantity of the chromatographically purified material was available for chemical analysis. With the details of separation which have been given in the above series of experiments it should be possible, even if it were time consuming, to obtain a new preparation of what appears to be a purified major promoter of germination of the spores of *P. eucalypti*. An Organic Chemist, from the data presented, could probably suggest means by which the isolation and purification procedure could be shortened and improved.
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APPENDIX No. 1

In addition, in the stomata penetrated, there was the production of a stoma
tal penetration towards stomatal openings. On the surface of the leaf, there was no evidence of a stimulus.

**Date of inoculation 10/8/64**

**Incubation period 5 days**

**Sample at 7 days after inoculation**

The spores on the leaf surface had swollen and germinated, two terminal germ tubes per spore were common. Occasional spores also showed germination from medial cells, such germ tubes were frequently shorter than the terminal germ tubes. This probably indicates, that the terminal cells of the spore germinated first. Some germ tubes already showed stomatal penetration. The Lugol's iodine stained the chloroplasts of the leaf cells a deep brown to black. The overall coloration of the leaf was blue-black probably indicating starch uniformly distributed throughout the leaf. The chloroplasts of the guard cells of the stomata, which had been penetrated, stained a brown black colour similar to those of non-penetrated stomata. There was no evidence of disorganisation of the chloroplasts of these cells.

**Samples at 9 and 11 days after inoculation**

The leaf pieces stained in L-Ph-Cotton Blue showed
stomatal penetration by more spores. In addition, in the stoma penetrated, there was the production of a stomatal appressorium under the surface, formed by the overhanging cuticle. The germ tubes appeared to spread randomly over the surface of the leaf. There was no evidence of a stimulus directing hyphal tip growth towards stomatal openings. On occasions a germ tube grew past a stoma and penetrated one at a distance. Sometimes a germ tube appeared to grow over the top of a stoma without infection occurring. The considerable glaucous covering on the leaves of E. bicostata, composed of numerous rods, may support the germ tube out of actual contact with the leaf surface. The penetration was normally carried out by a side branch, not the main germ tube tip. This branch was usually short and the appressorium formed did not limit the further growth of the main germ tube tip. As a result of this penetration behaviour the germ tube from a spore may penetrate more than one stoma.

It was observed that on occasions when two germ tubes of P. eucalypti penetrated the same stoma, both formed appressoria. The slide of the leaf pieces simmered in Lugol's iodine showed the same features as the samples taken at 7 days after inoculation i.e., no evidence of disorganisation of the chloroplasts of penetrated stomata, clear of chlorophyl.

Sample 12. Pleated received 12 hours darkness and 12 hours small areas — not as large as the interveinal islets — light before sampling.
Samples at 13 and 15 days after inoculation

The major histological change in these slides compared with previous ones was that intercellular hyphae had commenced to spread from the base of the stomatal cavities i.e., the penetration process was completed.

Samples at 17, 19 and 21 days after inoculation

Apart from further intercellular spread of the parasite, the major histological difference apparent in these slides was the bunching of hyphae of the pathogen in the substomatal cavities of stomata adjoining the stoma originally penetrated by the germ tube. The development of these bunched hyphae was followed in successive slides and they developed into the pycnidia of the fungus. This accords with the reference in the amended description of P. eucalypti that pycnidia are produced in the sub stomatal cavities (Walker, 1962). The leaf pieces stained in Lugol’s iodine revealed no signs of disorganisation of the photosynthetic and sugar to starch conversion mechanisms. The leaf tissues, including areas penetrated by intercellular hyphae, stained blue black as usual and the chloroplasts a brown black.

Sample at 23 days after inoculation

In this and subsequent samples, the leaf piece soaked in 95% methanol did not completely clear of chlorophyll.

Sample C. Plants received 21½ hours darkness and 7½ hours light before sampling.
remained green even when the leaf piece was in 95% Metha-
hol for 24 hours. When these leaf pieces were stained and
examined microscopically, these "green" areas were directly
below stomata which had been penetrated. The fungus was
spreading intercellularly and bunching in the substomatal
chambers of adjoining stomata, throughout these parts of the
leaf. Examination of these areas showed that the chloroplasts
were disorganised and a yellowish discoloration was evident.
The intercellular hyphae were spread widely from these "green"
areas into apparently healthy tissue. The leaf pieces stained in
Lugol's iodine showed that the chloroplasts in the "green" areas were broken down and
did not stain the typical black brown colour of those in the
healthy areas of the leaf. The chloroplasts in guard cells
of the stomata, overlying the "green" areas, some of which
did not disorganised and appeared normal.

Samples at 25 days after inoculation
The leaf samples collected were taken from the same
plants, but the light treatment prior to sampling was varied.
Sample A. Plants received 21 hours darkness.
Sample B. Plants received 21 hours darkness and 3½ hours
light
Sample C. Plants received 21 hours darkness and 7½ hours
light before sampling.
Histologically the leaf pieces stained in L-Ph-Cotton Blue, showed essentially the same features as those taken at 23 days except that the "green" areas were larger. The leaf pieces stained in Lugol’s iodine showed the following:

- **Sample A**: The leaf pieces stained light brown, only the interveinal is chloroplasts were light brown.

- **Sample B**: As for A, the hyphae of the parasite spreading.

- **Sample C**: The areas, corresponding to the "green" areas before staining, were light brown, remainder of the leaf was stained black brown. This was interpreted as indicating that starch production had ceased in the "green" areas. As in the sample taken at 23 days, the chloroplasts appeared normal except in the cells of the "green" areas where disorganisation was evident. An overall yellowish colour was apparent and no definite chloroplast wall was evident. Inter cellular hyphae were evident in the apparently living areas adjoining the "green" areas. On histological evidence it was decided that the latter were dying or dead. These observations are further evidence that this organism is a biotroph invading tissue which is still living and probably parasitising it although no evidence was available on this latter point.

**Samples at 26 and 28 days after inoculation**

The leaf pieces collected on these dates were essentially
Chlorotic areas on the leaf were now evident macroscopically. Small black fruits of the organism were scattered in these areas. When the leaf was cleared the "green" areas, which corresponded to the chlorotic areas, now occupied whole interveinal islets in some places. Histologically there was no evidence of the hyphae of the parasite spreading into the conducting tissue of the veins or the tissue of sclerenchyma and parenchyma surrounding the conducting stele.

It was also obvious that the fruits of the organism were developing from the bunched hyphae formed in the sub stomatal cavities. In cases where the developing fruits were causing swelling of the leaf, the guard cells of the stomata, through which the original penetration occurred, still contained apparently normal chloroplasts.

Sample at 30 days after inoculation

Cleared leaf pieces were essentially the same as those collected at 25 days except that sporulation was occurring from the substomatal fructifications. The ostiole of the pycnidium coincided with the stomatal pore. The cirri of spores was squeezed out through the opening between the guard cells.

Sample at 35 days after inoculation

The leaf pieces now showed heavy fructification with
numerous cirri protruding through stomata. The chloroplasts of the guard cells of these stomata were still intact they stained blue black in Lugol's iodine which suggests they were still carrying on starch synthesis. The individual infections of vein islets had spread only to the limits of the vein boundaries with no evidence of invasion of the vascular tissues. There was no evidence that the developing fruits actually ruptured the stomata the cirri protruded only through the stomatal pore.

In a particular experiment the chiffon pad was used for that experiment. In general, tests have shown that removal of oxygen prevents germination (Tuzet... 1926). The supply of oxygen present in an agar plate by a hanging drop culture frequently used in germination experiments, may become critical when the infection period is of 48 hours, as was necessary with Ceratocystis hirutus. It is also desirable with in vitro tests to ensure that there be little or no loss of water from the germination medium during the experiment otherwise, as may occur in more concentration, concentration of media is used as pH buffers etc. It was decided to use glass staining dishes as containers. These could be easily aseptically sterilized. When holding 6 ml of germination liquid they had a width of surface exposure of 1 cm2 on each of the germination medium. To
APPENDIX 2

Details of Materials and Methods, for "in vitro" germination experiments

The methods used have been in general the same throughout. If there has been a variation in a particular experiment the changes introduced are stated for that experiment.

In general terms it has been shown that removal of oxygen prevents spore germination. (Hart., 1926). The supply of oxygen to germinating spores in a hanging drop culture frequently used in germination experiments, may become critical when the germination period is of 48 hours, as was necessary with Phaeoseptoria eucalypti. It is also desirable with in vitro germinations that there be little or no loss of water from the germination medium during the experiment otherwise changes occur in spore concentration, concentration of materials used as pH buffers etc. It was decided to use crystal glass staining dishes as containers. These could be easily cleaned and sterilised. When holding 6 ml of germination medium they had a width of surface exposure of 3 times the depth of the germination medium.
prevent, or reduce, evaporation to a minimum the staining dishes were placed inside petri dishes which had a base covered with a 0.5 cm layer of wet vermiculite covered with a Whatmann No. 1 Filter paper. When the glass dishes were placed in these closed petri dishes even over a 96 hour period at 30°C these was no measurable loss of liquid from the dish.

The germination of the spores of non-obligate fungal parasites is likely to be affected by exogenous organic substrates, (Gottlieb, 1964). Glass distilled water was used to reduce exogenous substrates to a minimum e.g. in germination mediums, in the preparation of pH buffers etc. The germination dishes were cleaned by washing in warm water with detergent, rinsing with tap water, 70% ethanol (2 washings) glass distilled water (3 washings) and dried with paper tissues. Glass pipettes washed in tap water, absolute alcohol and distilled water (3 washings) were used for measuring all media, spore solutions, buffers etc. The spores used in the germination experiments were collected by brushing sporehorns from infected leaves into deionised water with a camel hair brush. This spore solution was then centrifuged at 3,000 revs/min. for 3 mins, the liquid decanted and the pellet of spores resuspended in glass distilled water. This procedure of washing the spores was repeated 3 times. The final spore suspension in glass
distilled water was stored in a cold room at 1° - 3°C. The spore solutions so collected contained spores of different ages and consequently of different germination potential (Cochrane, 1945). In a preliminary test with spores obtained from a 4 week old single spore culture it was found that the spores of the latter were just as variable in their germination as those collected from individual infected leaves. (These culture spores were probably also from pycnidia of various ages). The spore solutions from cultures were usually mixtures of spores mycelia and culture material and not very suitable for germination experiments. Finally the spores from artificial cultures did not shed into the water added to them and separate as well as did the spores collected from spore horns on infected leaves. For these reasons the spore solutions collected from infected leaves were used in the germination experiments.

The spore solution was shaken for two minutes by hand or an agitator to ensure mixing of the spores before samples were taken from the bulk store for germination. In addition air was blown through the spore store for 10 secs, with a pipette before taking aliquots for the experiments. When the germination dishes had been prepared, they were placed in the petri dishes with soaked vermiculite and filter paper
covering the base. These were covered and incubated for the treatment period at the selected temperature in the dark.

At the conclusion of the germination period the media were sampled. The particular medium was agitated with an eye dropper by blowing into the dish 10 times. An aliquot was taken from the dish and one drop of this was placed on a clean dry glass slide. The particular dish was agitated as before, a second aliquot taken and one drop placed on another clean dry glass slide. The drops of liquid were then dried off by placing the slides on a hot plate. When dry, one drop of Cotton-Blue-Lactophenol was placed on the dried drop of germinating medium. A thin coverslip was gently lowered onto the drop. The germinations were then ready for assessment.
TABLE No. 2/1 Germination of unwashed spores of Phaeoseptoria eucalypti in media made up of different concentrations of spore washings in distilled water, (each treatment contained .1 ml Citric Acid/Phosphate buffer pH 6.0 and .1 ml unwashed spores at 32,000 per ml).

<table>
<thead>
<tr>
<th>ml of distilled water</th>
<th>ml of spore washings</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>62.0 ± 1.59</td>
</tr>
<tr>
<td>0.4</td>
<td>0.1</td>
<td>57.4 ± 1.34</td>
</tr>
<tr>
<td>0.3</td>
<td>0.2</td>
<td>54.6 ± 1.57</td>
</tr>
<tr>
<td>0.2</td>
<td>0.3</td>
<td>53.5 ± 1.34</td>
</tr>
<tr>
<td>0.1</td>
<td>0.4</td>
<td>52.0 ± 1.36</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>51.5 ± 1.34</td>
</tr>
</tbody>
</table>
Germination of washed spores of *Phaeoseptoria eucalypti* in media made up of different concentrations of spore washings in distilled water (each treatment dish contained .1 ml Citric Acid/Phosphate buffer pH 6.0 and .1 ml of washed spore solution at a concentration of 32,000 spores per ml).

<table>
<thead>
<tr>
<th>ml of distilled water</th>
<th>ml of spore washings</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>56.6 ± 0.62</td>
</tr>
<tr>
<td>.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>.4</td>
<td>.1</td>
<td>56.0 ± 0.89</td>
</tr>
<tr>
<td>.3</td>
<td>.2</td>
<td>53.4 ± 0.42</td>
</tr>
<tr>
<td>.2</td>
<td>.3</td>
<td>51.8 ± 0.48</td>
</tr>
<tr>
<td>.1</td>
<td>.4</td>
<td>51.5 ± 0.27</td>
</tr>
<tr>
<td>0</td>
<td>.5</td>
<td>51.3 ± 0.53</td>
</tr>
</tbody>
</table>
TABLE No. 2/3 Germination of the spores of *Phaeoseptoria eucalypti* in relation to time from commencement of the experiment. (Germinations were carried out in media consisting of 0.4 ml distilled water, 0.1 ml Potassium hydrogen phthalate buffer pH 4.0 and 0.1 ml of washed spore solution concentration 32,000 per ml).

<table>
<thead>
<tr>
<th>Time after setting up germination experiment (in days)</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>18.0 ± 0.58</td>
</tr>
<tr>
<td>14</td>
<td>20.6 ± 0.64</td>
</tr>
<tr>
<td>18</td>
<td>26.0 ± 0.39</td>
</tr>
<tr>
<td>22</td>
<td>35.4 ± 0.38</td>
</tr>
<tr>
<td>24</td>
<td>38.4 ± 0.75</td>
</tr>
<tr>
<td>26</td>
<td>40.7 ± 0.69</td>
</tr>
<tr>
<td>28</td>
<td>41.7 ± 0.50</td>
</tr>
<tr>
<td>35</td>
<td>42.3 ± 0.34</td>
</tr>
<tr>
<td>45</td>
<td>42.6 ± 0.56</td>
</tr>
<tr>
<td>50</td>
<td>43.0 ± 0.84</td>
</tr>
<tr>
<td>60</td>
<td>43.8 ± 0.41</td>
</tr>
<tr>
<td>72</td>
<td>44.5 ± 0.47</td>
</tr>
</tbody>
</table>
Appendix 2

TABLE No. 2/4 Germination of spores of P. eucalypti in relation to time and method of storage.

<table>
<thead>
<tr>
<th>Time (in days)</th>
<th>Stored in solution at 0.5°-3.5°C</th>
<th>Stored dry at 0.5°-3.5°C</th>
<th>Stored dry in laboratory</th>
<th>Stored on living leaf of E. bicostata in laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65.3 ± 0.89</td>
<td>64.7 ± 1.28</td>
<td>65.1 ± 0.79</td>
<td>65.0 ± 0.79</td>
</tr>
<tr>
<td>7</td>
<td>67.0 ± 1.41</td>
<td>55.7 ± 0.75</td>
<td>46.9 ± 0.86</td>
<td>48.6 ± 1.40</td>
</tr>
<tr>
<td>14</td>
<td>65.6 ± 1.59</td>
<td>39.4 ± 1.49</td>
<td>36.0 ± 1.95</td>
<td>38.5 ± 0.73</td>
</tr>
<tr>
<td>21</td>
<td>65.3 ± 1.34</td>
<td>26.5 ± 0.68</td>
<td>24.0 ± 0.46</td>
<td>24.0 ± 0.46</td>
</tr>
<tr>
<td>28</td>
<td>66.2 ± 1.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>64.2 ± 0.98</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>63.9 ± 0.55</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>53.2 ± 0.72</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table No. 2/5

Germination of the spores of *Phaeoseptoria eucalypti* in relation to spore concentration in the germination medium.

<table>
<thead>
<tr>
<th>Calculated Spore concentration in germination medium</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>257,850</td>
<td>63.3 ± 1.08</td>
</tr>
<tr>
<td>128,260</td>
<td>71.9 ± 1.57</td>
</tr>
<tr>
<td>67,462</td>
<td>74.7 ± 1.59</td>
</tr>
<tr>
<td>32,005</td>
<td>71.8 ± 1.45</td>
</tr>
<tr>
<td>16,110</td>
<td>62.7 ± 1.95</td>
</tr>
<tr>
<td>7,941</td>
<td>62.2 ± 2.32</td>
</tr>
<tr>
<td>4,058</td>
<td>52.2 ± 1.97</td>
</tr>
<tr>
<td>2,120</td>
<td>42.4 ± 0.99</td>
</tr>
<tr>
<td>1.0</td>
<td>52.7 ± 1.94</td>
</tr>
<tr>
<td>0.5</td>
<td>63.1 ± 1.47</td>
</tr>
<tr>
<td>0.0</td>
<td>68.6 ± 1.35</td>
</tr>
<tr>
<td>0.5</td>
<td>60.6 ± 1.47</td>
</tr>
<tr>
<td>0.0</td>
<td>61.9 ± 1.30</td>
</tr>
<tr>
<td>0.5</td>
<td>69.1 ± 0.87</td>
</tr>
<tr>
<td>0.0</td>
<td>66.1 ± 1.95</td>
</tr>
<tr>
<td>0.5</td>
<td>63.2 ± 0.96</td>
</tr>
<tr>
<td>0.0</td>
<td>63.9 ± 0.84</td>
</tr>
<tr>
<td>0.5</td>
<td>29.0 ± 0.84</td>
</tr>
<tr>
<td>0.0</td>
<td>55.5 No buffer but 1 ml distilled water added 66.1 ± 1.26</td>
</tr>
</tbody>
</table>

Buffer solution: 0.01 M NaCl/0.01 M citric acid/KOH or NaOH.
Appendix 2

**TABLE No. 2/6**  Germination of spores of *P. eucalypti* over a range of pH values using various buffering materials. (In each .1 ml of appropriate buffer was added to .4 ml of distilled water and .1 ml of washed spore solution).

<table>
<thead>
<tr>
<th>pH</th>
<th>Citric Acid/Phosphate</th>
<th>Buffer Solution</th>
<th>Mean ontogenetic germination score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Potassium Hydrogen</td>
<td>HCL/K CL</td>
</tr>
<tr>
<td>1.0</td>
<td>73.25 ± .32</td>
<td>28.2 ± .77</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>80.5 ± 2.19</td>
<td>34.5 ± 1.10</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>73.0 ± 1.73</td>
<td>43.3 ± 1.21</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>52.6 ± 1.21</td>
<td>42.0 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>67.2 ± 1.73</td>
<td>52.7 ± 1.14</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>65.1 ± 1.47</td>
<td>68.6 ± 1.35</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>68.5 ± 1.67</td>
<td>72.1 ± 1.90</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>60.4 ± 1.47</td>
<td>76.5 ± 1.49</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>61.9 ± 1.30</td>
<td>68.9 ± .74</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>No buffer but .1 ml distilled water added 66.1 ± 1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>66.1 ± 1.95</td>
<td>66.0 ± 1.06</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>63.2 ± .96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>43.9 ± .84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>29.0 ± .84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Germination of spores of *Phaeoseptoria eucalypti* over a range of pH values using two buffering materials. (.4 ml of appropriate buffer, .1 ml distilled water and .1 ml washed spore solution concentration 32,000 per ml used in each treatment). These results are for comparison with those given in Table 2/6 where the same buffers were used at a lower concentration.

<table>
<thead>
<tr>
<th>pH</th>
<th>Citric Acid/Phosphate</th>
<th>Potassium Hydrogen phthalate/NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>75.25 ± .52</td>
<td>27.5 ± .64</td>
</tr>
<tr>
<td>2.6</td>
<td>80.5 ± 2.19</td>
<td>27.0 ± 0.0</td>
</tr>
<tr>
<td>3.0</td>
<td>75.0 ± 1.73</td>
<td>28.0 ± .57</td>
</tr>
<tr>
<td>3.4</td>
<td>85.5 ± .63</td>
<td>28.0 ± .57</td>
</tr>
<tr>
<td>4.0</td>
<td>36.5 ± 1.94</td>
<td>37.8 ± .64</td>
</tr>
<tr>
<td>4.3</td>
<td>55.5 ± 2.56</td>
<td>52.5 ± 1.66</td>
</tr>
<tr>
<td>4.8</td>
<td>31.25 ± 1.12</td>
<td>56.3 ± .71</td>
</tr>
<tr>
<td>5.0</td>
<td>29.5 ± .87</td>
<td>59.3 ± 1.83</td>
</tr>
<tr>
<td>5.3</td>
<td>25.0 ± 0</td>
<td>48.3 ± 2.56</td>
</tr>
</tbody>
</table>
Effect of Temperature on the germination of spores of Phaeoseptoria eucalypti. (These germinations were carried out in seed germination cabinets with individual temperature control housed at C.S.I.R.O. Division of Plant Industry. The temperature control of these cabinets proved to be somewhat inaccurate).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Mean ontogenetic germination score</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>27 ± 0.85</td>
</tr>
<tr>
<td>13.9</td>
<td>48.6 ± 1.18</td>
</tr>
<tr>
<td>17.5</td>
<td>58.2 ± 1.95</td>
</tr>
<tr>
<td>21.3</td>
<td>73.3 ± 1.04</td>
</tr>
<tr>
<td>22.7</td>
<td>64.6 ± 2.18</td>
</tr>
<tr>
<td>26.7</td>
<td>64.8 ± 1.69</td>
</tr>
<tr>
<td>27.2</td>
<td>62.2 ± 1.03</td>
</tr>
<tr>
<td>31.5</td>
<td>52.5 ± 1.66</td>
</tr>
<tr>
<td>35.3</td>
<td>28.3 ± 0.61</td>
</tr>
</tbody>
</table>
### TABLE No. 2/9

Effect of Temperature on the germination of spores of Phaeoseptoria eucalypti. (These germinations were carried out in cabinets with individual temperature control set in a cold room at the Botany Dept, A.N.U.).

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Mean number of germ tubes per group of 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.25 ± 0.46</td>
</tr>
<tr>
<td>10</td>
<td>9.8 ± 0.53</td>
</tr>
<tr>
<td>15</td>
<td>26.9 ± 0.56</td>
</tr>
<tr>
<td>20</td>
<td>20.5 ± 0.59</td>
</tr>
<tr>
<td>25</td>
<td>20.0 ± 0.46</td>
</tr>
<tr>
<td>30</td>
<td>21.13 ± 0.63</td>
</tr>
<tr>
<td>35</td>
<td>1.33 ± 0.18</td>
</tr>
<tr>
<td>40</td>
<td>5.52 ± 0.68</td>
</tr>
<tr>
<td>45</td>
<td>26.49 ± 0.80</td>
</tr>
<tr>
<td>50</td>
<td>52.9 ± 1.16</td>
</tr>
</tbody>
</table>

Note: Temperatures were measured using an electronic thermometer.
APPENDIX 3

The areas of microscopic leaves of *E. bicostata* used in the various experiments were determined graphically; measurements were also made of width and breadth of these leaves. These data, along with measurements of leaf area, were used to prepare a chart and the area under this chart. The intercepts of this chart were plotted to obtain a mean area table for least squares. Only the least area table was prepared to cover a range of leaf area.

Percentage of the water soluble fraction of the gemination medium

<table>
<thead>
<tr>
<th>Percentage of the water soluble fraction of the germination medium</th>
<th>Mean ontogenetic germination score for 25 spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.66</td>
<td>35.0 ± 0.68</td>
</tr>
<tr>
<td>0.33</td>
<td>34.9 ± 0.80</td>
</tr>
<tr>
<td>0.16</td>
<td>37.0 ± 0.57</td>
</tr>
<tr>
<td>0.00</td>
<td>52.9 ± 1.18</td>
</tr>
</tbody>
</table>
Construction of Leaf area alignment chart.

The areas of some 400 leaves of *E. bicostata* used in the various experiments were determined graphically; measurements were also made of the length and breadth of these leaves. These data were then plotted on a three way alignment chart, the length and breadth measurements on the vertical axes and the area measurement on the base of the chart. A curve was fitted to the resulting points by method of least squares. From this curve a leaf area table was prepared to cover a range of leaf sizes using the intercept values of leaf dimensions on the alignment curve.