Trans. Br. mycol. Soc. **82** (2), 257–273 (1984) *Printed in Great Britain*

LEAF SPOT OF *EUCALYPTUS* CAUSED BY *AULOGRAPHINA EUCALYPTI*

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Aulographina eucalypti causes a distinctive circular leafspot on *Eucalyptus* that usually develops only partly through the lamina. The disease was commonly found on many *Eucalyptus* species from both major subgenera, and also on the related *Angophora costata*. The fungus grew slowly in culture. Leaf penetration occurred from appressoria directly through the cuticle and the fungus formed both surface and sub-cuticular colonizing hyphae. Sporophores were formed on the lesion surface. Lesions observed in the field developed very slowly, with pseudothecium development occurring 5 months after initial appearance of a tiny lesion. Ascospore release was most prolific on lesions wetted and kept in the light. The lesion and fungus apparently become a sink for photosynthate from healthy parts of infected leaves.

Only two major epidemics of a leaf pathogen in mature *Eucalyptus* have been reported. In 1973 extensive defoliation of *E. obliqua* in several valleys in north-western Tasmania was attributed mainly to *Aulographina eucalypti* (Cke & Massee) v. Arx & Müller (C. Palzer, pers. comm.). In 1974 over 4200 ha of good-quality, mature *Eucalyptus nitens* on the Errinundra Plateau, Victoria, were moderately to severely defoliated (Neumann & Marks, 1976). The major pathogen was again considered to be *A. eucalypti*.

A. eucalypti is a loculoascomycete associated with a leaf spot found commonly on many species of *Eucalyptus*. On the basis of association, Müller & von Arx (1962) described the pycnidial fungus *Thyrinula eucalyptina* Petrak & Syd. as the conidial state of *A. eucalypti*. They also concluded that *Aulographum eucalypti* Cke & Massee, *Lembosiopsis eucalyptina* Petrak & Syd. and possibly *L. australiensis* Hansf. were synonyms of *Aulographina eucalypti*. The former was reported from *E. dives*, *E. niphophila* and *E. stellulata* (Anon., 1959). *L. eucalyptina* was reported from foliage of *E. delegatensis* (*E. giganteus*) and *E. globulus* in South Africa (Petrak & Sydow, 1924) and *L. australiensis* was reported from foliage of *E. cosmophylla* and *E. marginata* (Hansford, 1954). More recently *A. eucalypti* has been reported on foliage of *E. agglomerata* in Olney State Forest, N.S.W. (Gerrettson-Cornell & Dowden, 1977). *A. eucalypti* has also been reported from several eucalypt species, including *E. coccifera*, in the United Kingdom (Spooner, 1981). The disease caused by *A. eucalypti* has been referred to as corky leaf spot by Marks, Fuhrer, Walters & Huebner (1982) who have given an outline of symptoms and host range.

Here we describe symptoms and development of the leaf spotting disease caused by *A. eucalypti*, and report studies of host range, sporulation, culturing, pathogenesis and physiological effects of the fungus on its host.

MATERIALS AND METHODS

Symptoms, disease development and host range

Symptoms were described from diseased leaves and branches collected mainly at Narbethong, Victoria. In the study of host range, diseased foliage was collected from many eucalypt species in a wide range of habitats, including plantations and gardens. Sections were cut for microscopic examination using a sledge microtome and a 'Frigistor' freezing stage and were mounted in lactophenol cotton blue or 30% glycerol. Alternatively, leaves were fixed in 50% formal acetic alcohol. They were then cleared and stained using a modification of Nair's (1976) method for the periodic acid Schiff (PAS) reaction, although lactophenol cotton blue was not used and the PAS reagents were prepared following Gurr (1973). Leaf sections were left in Shiff's reagent for up to 30 min.

Leaf pieces were prepared for scanning electron microscopy using the thiocarbohydrazide-mediated osmium binding method of Kelley, Dekker & Bluemink (1975) except that the tissue was fixed under vacuum and 0·1 M phosphate buffer was used instead of cacodylate. The specimens were examined in a Siemens ETEC Autoscan scanning electron microscope.

In June 1980, 8 leaves on saplings of *E. obliqua* at Narbethong were tagged. At monthly intervals

lesions were counted, the diameters of all lesions on the leaves were measured and the proportion of lesions with pycnidia, or pycnidia and pseudothecia, was recorded.

In studies of variability of the pathogen, dimensions of fungal structures were measured on lesions collected from *E. regnans* at two sites (Narbethong in Central Victoria and Beech Forest in South Western Victoria), *E. obliqua* and *E. baxteri* at Narbethong, *E. globulus* at two sites (Nowa Nowa in eastern Victoria and Hawaii) and *Angophora costata*.

Culturing

Small pieces of leaf, usually including both necrotic and healthy tissue, were surface sterilized with 70% ethanol for up to 10 min, washed in two rinses of distilled water and incubated on Water Agar. Emerging hyphae were transferred to various nutrient media.

Ascospores were deposited on Water Agar from moistened lesions attached to lids of Petri dishes. Single and multiple ascospore cultures were established by transferring spores to various nutrient media in Macartney bottles. Isolates grown from ascospores were established on various media (Malt Agar, Potato Dextrose Agar and Water Agar), with and without 1% Chloramphenicol and eucalypt leaf pieces which had been sterilized by gamma-irradiation (2·5 megarad for 8 h). Cultures were incubated at room temperature (18–25 °C) and exposed to daylight on the laboratory bench. Growth rates of single ascospore cultures were determined in Petri dishes sealed with plastic tape.

A pycnidiospore suspension was prepared by immersing lesions bearing mature pycnidia in distilled water for several minutes. The suspension was spread on various nutrient media and pycnidiospores were examined for germination.

Sporulation

To determine the effect of relative humidity on ascospore release, lesions bearing pseudothecia were attached with tape to microscope slides resting on V-shaped glass rods above a range of saturated salt solutions in Petri dishes (Anon., 1968). Lesions were allowed to dry in the air for 7 h before being placed in Petri dishes, which were incubated in the dark at 20° for 48 h. Microscope slides were then attached to the lids of the Petri dishes, 4 mm above the lesions, and the dishes were sealed with plastic tape and left for a further 48 h in the dark at 20°. Ascospores trapped on these slides were stained with lactophenol cotton blue and counted. The total length of pseudothecium on each lesion was measured and spore counts were expressed per unit length of pseudothecium. The effect of different periods of leaf wetness on ascospore release was also studied. Lesions bearing pseudothecia were soaked in deionized water for varying periods and were then blotted with tissue paper to remove free water and placed on moist filter paper in Petri dishes. There were 3 replicates for each treatment. In one experiment the dishes were left uncovered. In another the filter paper was moistened and the dishes were covered to keep lesions moist. Microscope slides were mounted 3 mm above lesions in the Petri dishes and were changed periodically. Ascospores shed on to the microscope slides during each interval were counted.

A similar apparatus was used to determine the effect of temperature on ascospore release. Lesions, after initial air-drying for 7 h, were moistened by spraying with distilled water. After an initial incubation for 48 h at 20°, a trap slide was attached to the lid of each Petri dish 4 mm above the lesions and the dishes were incubated for 48 h in the dark at a range of temperatures. In all experiments 5 lesions were mounted in each Petri dish and 3 dishes were used for each treatment.

To study the effect of light on ascospore release, batches of five lesions cut from leaves of *E. obliqua* were kept for 12 h at 17° in the dark or light (90–100 μEi m^{-2} s^{-1}) on either moist or dry filter paper in Petri dishes. Ascospore release during the 12 h period was determined on microscope slides positioned 4 mm above lesions. Some of the lesions from the above treatments were soaked for 30 min in distilled water. Excess free water was removed with tissue paper and the lesions were incubated in the dark or light as above. Ascospore release was again recorded at hourly intervals.

Sporulation was monitored in the field by attaching microscope slides to lesions on intact leaves. The slides were removed at intervals and examined with a microscope for the presence of ascospores.

To determine the discharge distance of ascospores, lesions with pseudothecia were attached to the lids of Petri dishes containing various depths of 1·5% Water Agar. The lesions were sprayed with distilled water and the Petri dishes were inverted and incubated in the dark at 20° for 48 h after which time ascospores on the agar were counted.

For later pathogenicity and fungicide tests, spores were collected using an apparatus developed by R. F. Park (pers. comm.). Leaves were soaked for 30 min and clamped into four racks about 10 cm × 40 cm which were then held vertically with 2 cm between each rack in a perspex box. Air,

drawn through the box with a vacuum pump attached to the base, passed through several fine holes in a perspex plate inserted just above the base. Ascospores impacted on to glass coverslips placed just below these holes and could be washed off to give a spore suspension.

Pathogenesis

Small moisture chambers were prepared from plastic Petri dishes. A hole in the side of the dish allowed a leaf attached to a seedling to be housed in the dish where it was kept moist. Lesions bearing pseudothecia were attached to the lid so that ejected ascospores were deposited on the leaf. These plants were kept in a moist chamber for 2 d after inoculation before being transferred outside into a shade house.

Ascospore suspensions were prepared by attaching moist sporulating lesions to the lids of Petri dishes above cellophane on Water Agar or above empty bases. Deposited ascospores were washed into a vial using a weak Tween 20 solution (0·2 μl ml^{-1}) and were concentrated by centrifugation at 5000 ***g*** for 10 min. The concentration of ascospores was determined with a haemocytometer and their viability was tested on Water Agar. Spore suspensions were then sprayed or painted with a camel hair brush on to seedlings which were then kept in a dew chamber (12 h dark/12 h light, 30–70 μEi m^{-2} s^{-1}; 15°) for up to 14 d before being transferred to a shade house. Seedlings inoculated in the same way were sprayed with distilled water, covered with plastic bags, and kept in a growth room (65% r.h., 450 μEi m^{-2} s^{-1}, 18°) for several days. Bags were removed for 6–10 h each day for several days before the plants were uncovered permanently and transferred to a shade house.

Sporulating moist lesions were attached with vaseline in the bases of thin, clear plastic blood-testing blisters, which were then attached to leaves with a paper clip. Seedlings bearing several such blisters were then placed in a dew chamber (12 h dark/12 h light, 30–70 μEi m^{-2} s^{-1}, 15°) for up to 15 d. They were kept for brief periods in a growth room, alternating with periods in a dew chamber, to harden the plants before they were transferred to a shade house. Seedlings inoculated in the same way were sprayed with distilled water, covered with plastic bags and kept for several days in a growth room as described above. Bags were removed for 6–10 h each day for several days before the plants were uncovered permanently and transferred to a shade house.

Infection processes were studied microscopically on leaves removed at various intervals after inoculation. Leaf pieces were fixed in 50% formal acetic alcohol for 1 h, stained with lactophenol cotton blue or cleared and stained following the method of Bevege (1968). Alternatively leaves were fixed as previously described and stained with the PAS reaction with leaf sections being left in Shiff's reagent for up to 1 h.

Physiological studies

Anthocyanin was extracted from the purple margins of lesions in cooler months following the method of Harborne (1973). One gram of leaf discs, 9 mm diam, was cut from diseased mature leaves of *E. obliqua*. The tissue was ground in 7 ml methanol with 1% hydrochloric acid in a mortar with a pestle. A similar extract was made from healthy leaves. Extracts were run with cyanidin-3-glucoside standard on Kodak Eastman cellulose paper in n-butanol-acetic acid-water (4 : 1 : 5).

For autoradiographic studies of incorporation and distribution of ^{14}C in leaves, branches of *E. obliqua* were collected in the field, cut under water and brought back to the laboratory in a bucket covered with a polythene bag. Branches were exposed to light for 1 h and three diseased and three healthy leaves were cut from the branches under water and arranged in a glass box with only their petioles in water. Leaves were exposed for 30 min to $^{14}CO_2$, released from 1 ml of 10 mCi Ba $^{14}CO_3$ by a drop of 1 M acetic acid, in a sealed glass box (2·276 l) exposed to a light intensity of 100–150 μEi m^{-2} s^{-1} at 22°. The box contained 128 μg CO_2 to which 88 μg $^{14}CO_2$ was added. Thirty minutes after the box was opened, leaves were removed and placed on Agfa-Gevaert Osray M3-DW film and clamped between two glass plates at 5° for 24 h.

For extraction and measurement of unhydrolysed sugars in leaf tissue 5 g of leaf discs, 8 mm diam, were cut from healthy and diseased regions of mature *E. obliqua* leaves. Discs from diseased regions contained a complete lesion plus the purple margin and a small amount of green tissue. Tissue was finely chopped with a razor blade and added to 20 ml 70% ethanol which was boiled for 5 min. The extract was decanted and the tissue was re-extracted twice with fresh lots of 70% ethanol. Extracts were combined, evaporated to dryness and redissolved in 10 ml distilled water. Six drops of lead acetate and four drops of potassium dihydrogen orthophosphate were added to precipitate proteins and absorb tannins and other colouring agents. The extract was then centrifuged at 400 ***g*** for 10 min and the supernatant was decanted and made up to 50 ml with distilled water. The amount of unhydrolysed sugar in the extracts was determined by the picric acid method. A 4 ml sample was added to 4 ml

picrate solution (36 g picric acid, 500 ml 1% NaOH, made up to 1 l with distilled water) and 1 ml 20% aqueous Na_2CO_3 (anhydrous w/v). Samples were placed in a boiling water bath for 10 min, cooled and the absorbance at 510 nm measured in a spectrophotometer. The extraction was replicated three times.

To determine the effect of various fungicides on ascospore germination, 10 ml lots of fungicide dilutions were added to 4% phosphate buffered Water Agar (pH 6·5) to give final concentrations from 0 to 250 μg ml^{-1} of active ingredient. Fungicides tested were benomyl (Benlate, 50% a.i.), Captan (83% a.i.) and copper oxychloride (12·5% a.i.). Ascospores were collected in the spore trap described above, washed into suspension with a freshly prepared Tween 80 solution (0·4 μl ml^{-1}) and adjusted to a concentration of 5×10^4 spores ml^{-1}. The spore suspension was applied with a wire loop to four marked spots on each Petri dish of agar which was then incubated at 18° under a light intensity of 60–90 μEi m^{-2} s^{-1}. After 48 h, germination of at least 50 ascospores per spot was determined.

RESULTS

Symptoms, disease development and host range

A. eucalypti was identified on lesions of leaf laminae, petioles, small branches, fruits and leaf litter of *E. obliqua* and *E. regnans* at Narbethong and *E. nitens* on the Errinundra Plateau in Victoria. It was also commonly found on the smooth bark of *E. regnans*. On leaves it was associated with circular necrotic lesions, often slightly raised and corky, occurring on either the adaxial or abaxial leaf surface but rarely developing completely through the lamina (Figs 1, 2). Foliar lesions were 2–15 mm diam and were often restricted by a saucer-shaped cork cambium formed under and around the necrotic spot (Fig. 3). During cooler months (May to October) a purple margin, 0·4–2 mm wide, developed around lesions and was frequently visible on both leaf surfaces (Fig. 4). In some cases the necrotic region was cracked. Lesions on petioles were extensively cracked (Fig. 5). Small, circular, black, pimple-like pycnidia and black, elongate, often branched pseudothecia, which opened by a split along their length, were formed usually in roughly concentric rings on the surface of lesions (Figs 2, 3, 4, 6, 7). Asci were clavate and bitunicate with two-celled ascospores generally in two rows, and were surrounded by paraphyses (Fig. 8). Centrally located mature sporophores were often surrounded by smaller, apparently immature sporophores. On very young lesions pycnidia were always formed before pseudothecia; on older lesions both sporophores usually occurred on the one lesion. Necrotic epidermal cells in the centre of lesions stained heavily with the PAS reaction. In younger lesions this region was surrounded by concentric areas of non-staining and staining discoloured epidermal cells. In older lesions the central necrotic region was surrounded by alternating areas of discoloured and necrotic epidermal cells.

Lesions on leaves were associated with two types of hyphae. Melanized septate hyphae grew over the leaf surface and frequently extended well beyond the necrotic zone (Figs 9, 10). In some cases hyphae appeared to pass through stomata. Surface hyphae often anastomosed and sometimes showed signs of degeneration. Melanized hyphae were often present in substomatal cavities and stomatal pores of the necrotic area. A subcuticular stroma of densely packed non-melanized hyphae, 1–3 layers thick, was present in the necrotic region and extended to the perimeter of the necrotic area (Fig. 9). The stromatic hyphae had a greater diameter than surface hyphae and had melanized septa (Fig. 11). They appeared to diverge around stomata and on rare occasions were found inside epidermal cells. Sub-cuticular hyphae often appeared to emerge either through stomata or directly through the cuticle on to the lesion surface (Fig. 12). On occasions hyphae also grew into necrotic mesophyll tissue.

Development of lesions on marked leaves of *E.*

Figs 1–7. *Aulographina eucalypti.*

Fig. 1. Adult leaf of *E. obliqua* heavily infected with *A. eucalypti*. Bar = 10 mm.

Fig. 2. Leaf spot on *E. obliqua*, showing pseudothecia. Bar = 1 mm.

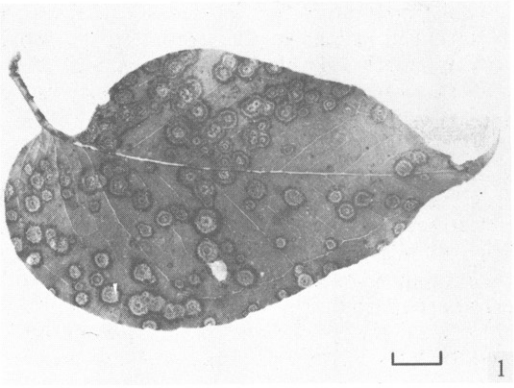
Fig. 3. Transverse section through leaf spot on *E. obliqua*, showing healthy tissue, cork cambium (arrowed) and necrotic tissue below a pseudothecium. Bar = 10 μm.

Fig. 4. Adult leaf of *E. obliqua* heavily infected showing purple margins formed around leaf spots in winter. Bar = 10 mm.

Fig. 5. Lesion caused by *A. eucalypti* on petiole of *E. nitens*. Bar = 10 mm.

Fig. 6. Pimple-like pycnidia and elongate pseudothecia on leaf spot surface. Bar = 100 μm.

Fig. 7. Scanning electron micrograph showing pycnidium, pycnidiospores (arrowed) and surface hyphae. Bar = 15 μm.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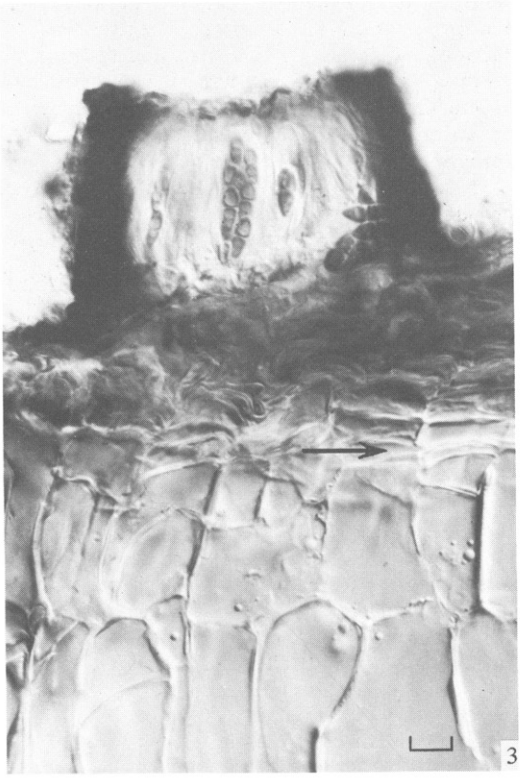
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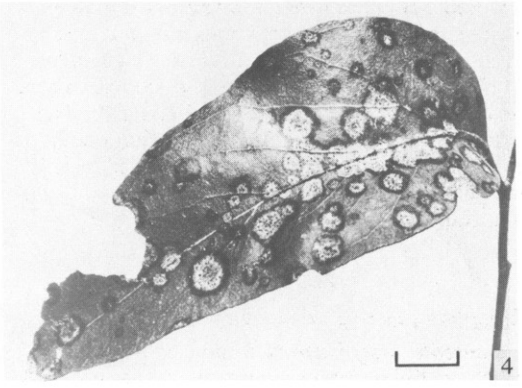
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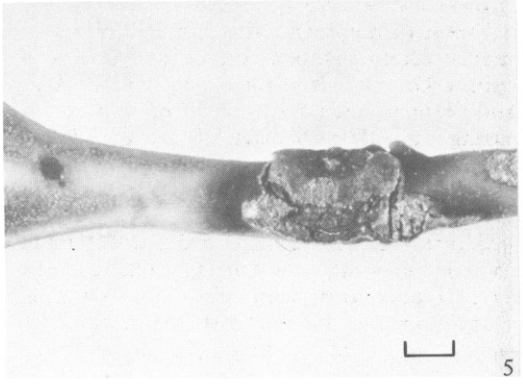
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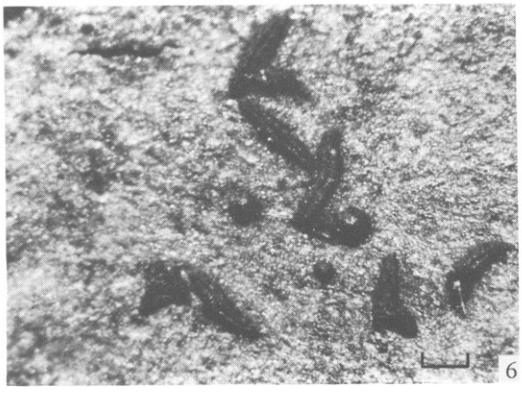
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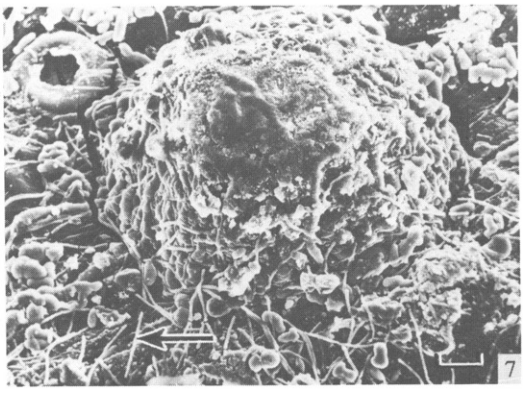
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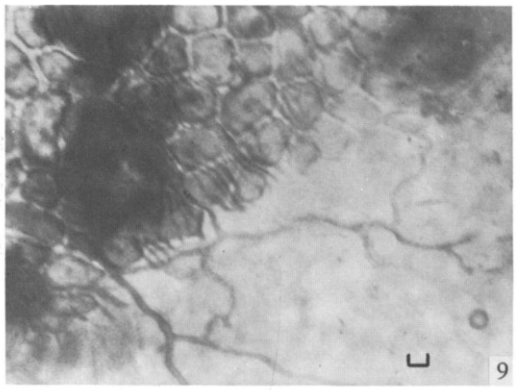

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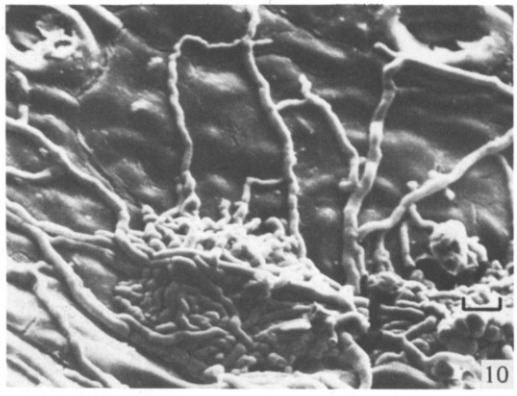

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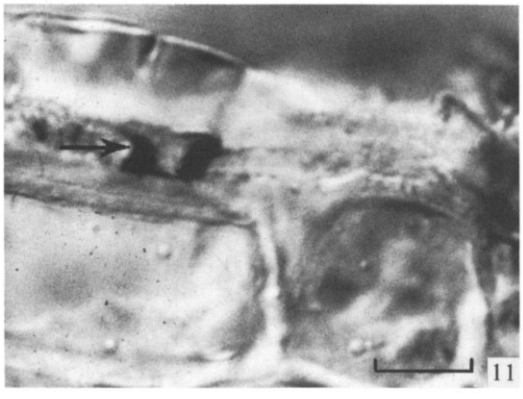

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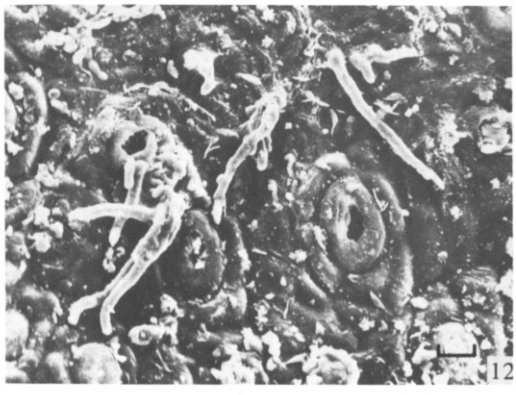

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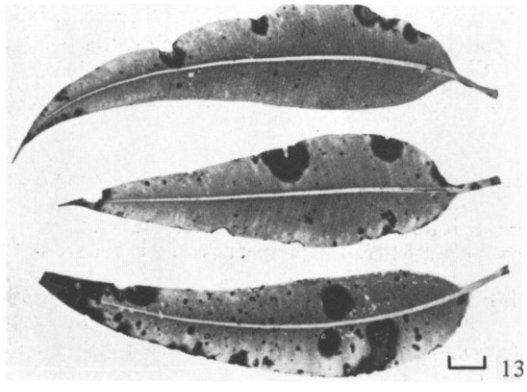

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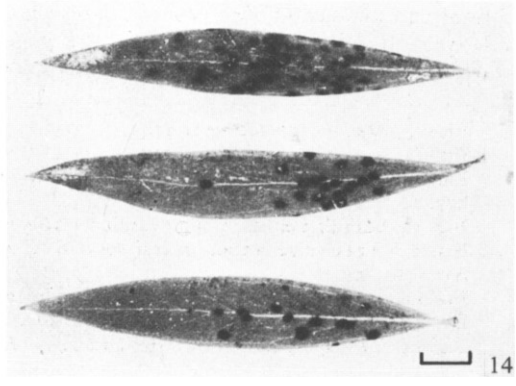

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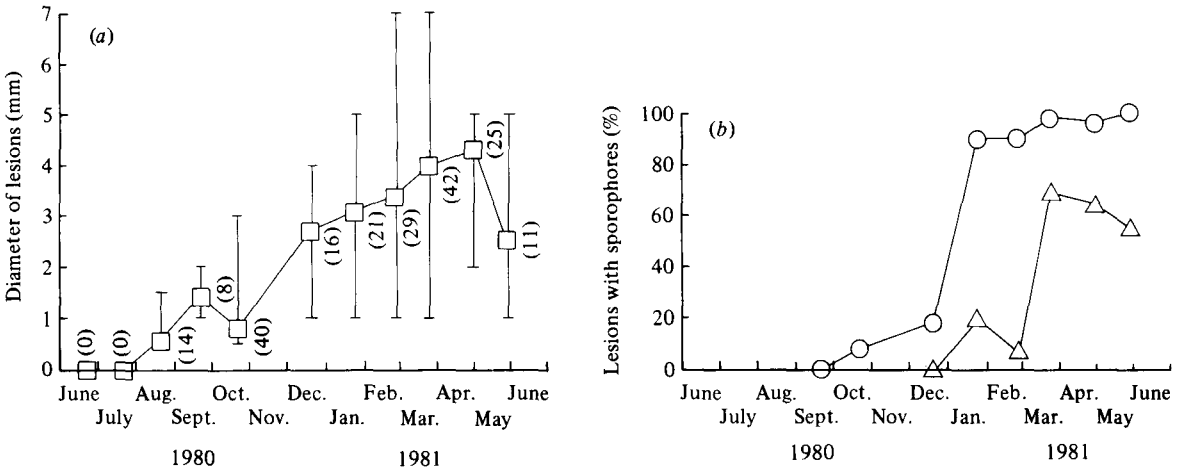


Fig. 15. Mean diam (with range) of lesions (*a*), and proportion of lesions with pycnidia (○) or pycnidia and pseudothecia (△) (*b*), measured over a period of time on 3 marked leaves of *E. obliqua* with no visible lesions at commencement of observations. Total numbers of lesions observed at monthly readings are given in brackets.

obliqua in the field at Narbethong is shown in Figs 15 and 16. Three leaves (Fig. 15) had no visible lesions in June 1980 when observations commenced. On these leaves lesions first became visible in August (mean diam 0·5 mm) and were still growing 8 months later in March 1981 (mean diam 4 mm). Pycnidia were first observed in October, 2 months after lesions became visible and pseudothecia were first observed in January 1981, 5 months after lesions became visible. Five leaves (Fig. 16) had very small lesions (mean diam 0·7 mm) in June 1980. These lesions had expanded to 4 mm diam by January 1981 (7 months later); thereafter they appeared to expand no further. Pycnidia were first observed on these lesions in July, 1 month after observations had begun, and pseudothecia were first observed in October.

A. eucalypti was identified from leaf spots on a range of *Eucalyptus* species from both major subgenera (Table 1). The fungus was also identified on stems of *E. saligna* and foliage of *E. globulus* from Maui, Hawaii (C. S. Hodges, pers. comm.) and on foliage and petioles of *Angophora costata*, a close relative of *Eucalyptus*. Foliar lesions on *A. costata* differed from those on *Eucalyptus* in occurring right through the leaf. Pycnidia and pseudothecia occurred on one or other leaf surface in concentric rings, although they were more common on the abaxial surface. They rarely occurred on both surfaces of a lesion. Foliar lesions were commonly surrounded by irregular pigmented zones of leaf tissue, with a red zone adjacent to necrotic tissue, a yellow zone adjacent to healthy tissue and an orange zone in between (Fig. 13). Mature lesions with sporophores were 2·2–9·5 mm diam (including the pigmented zone which was 0·2–1·2 mm wide). Smaller, similar lesions lacking sporophores were also seen.

There were no marked differences in dimensions between the isolates from the various *Eucalyptus* species and *A. costata* (Table 2).

The isolates also did not vary greatly with respect to pseudothecium length (range of means 310–462 μm), pseudothecium width (92–133 μm), ascus length (31–38 μm), ascus width (10–12 μm), paraphysis length (29–47 μm), pycnidium diam (80–94 μm) and conidiophore length (5–7 μm).

A very similar leaf spot and fungus, *Lembosina persooniae* Swart, were found on *Persoonia sylvatica* L. Johnson (Fig. 14).

Figs 8–14. *Aulographina eucalypti*

Fig. 8. Bitunicate asci. Bar = 10 μm.

Fig. 9. Micrograph of leaf spot margin showing irregular surface hyphae and regular sub-cuticular stromatic hyphae. Bar = 10 μm.

Fig. 10. Scanning electron micrograph showing surface hyphae and pseudothecia initials. Bar = 10 μm.

Fig. 11. Transverse section of leaf spot showing sub-cuticular hypha with melanized septum (arrowed). Bar = 10 μm.

Fig. 12. Scanning electron micrograph showing emergence of hyphae from stoma and cuticle. Bar = 10 μm.

Fig. 13. Leaf spots on *A. costata*. Bar = 10 mm.

Fig. 14. Leaf spots caused by a similar fungus, *Lembosina persooniae*, on *P. sylvatica*. Bar = 10 mm.

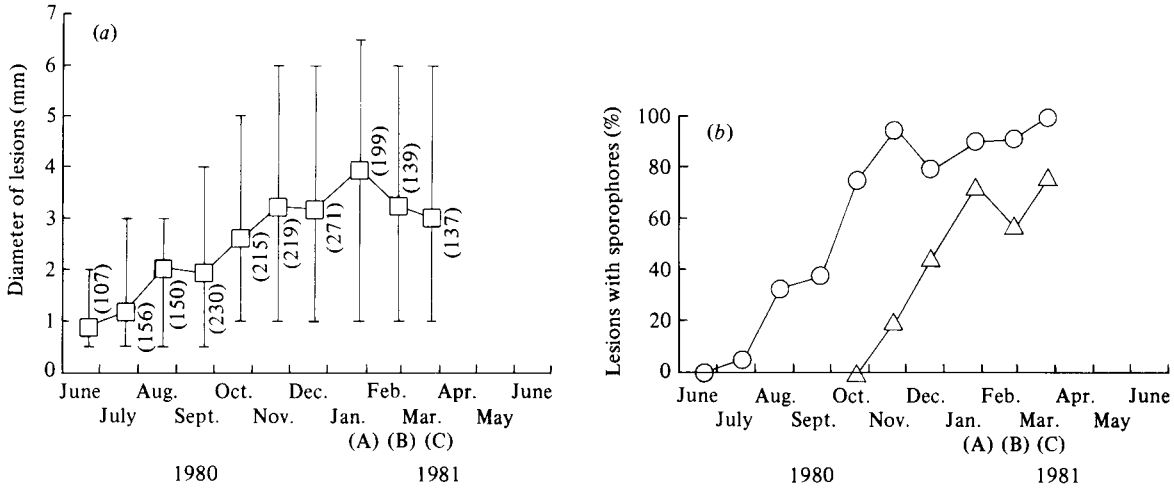


Fig. 16. Mean diam (with range) of lesions (*a*), and proportion of lesions with pycnidia (○) or pycnidia and pseudothecia (△) (*b*), measured over a period of time on 5 marked leaves of *E. obliqua* with small lesions at commencement of observations. Total numbers of lesions observed at monthly readings are given in brackets. (A) Leaf no. 8 fallen; (B) leaf nos. 6, 7 fallen; (C) leaf nos. 4, 5 fallen.

Table 1. *Recorded hosts of* A. eucalypti

Host	Location	Status*	Reference
E. (*M.*)† *agglomerata* Maid.	N.S.W.		Gerrettson-Cornell & Dowden (1977)
E. (*M.*) *approximans* Maid.	A.C.T.	O	‡
E. (*M.*) *baxteri* (Benth.) Maid. & Blakely	Vic.	N,C	‡
E. (*S.*) *bridgesiana* R. T. Bak.	Vic.	N,R	‡
E. (*S.*) *caseia* Benth.	A.C.T.	O,R	‡
E. (*S.*) *camaldulensis* Dehnh.	Vic.	P,R	‡
E. (*S.*) *cladocalyx* F. Muell.	Vic.	P,R	‡
E. (*S.*) *coccifera* Hook.	Devon, U.K.		Spooner (1981)
E. (*M.*) *consideniana* Maid.	Vic.	N,R	‡
E. (*S.*) *cypellocarpa* L. Johnson	Vic.	N,R	‡
E. (*S.*) *dalrympleana* Maid.	A.C.T.	N,R	‡
E. (*M.*) *delegatensis* R. T. Bak.	S. Africa		Petrak & Sydow (1924)
	A.C.T.		Burdon & Chilvers (1974)
	Tas.	P,C	‡
	Tas., Vic.	N,C	‡
E. (*M.*) *dives* Schau.	S. Aust.		Anon. (1959)
	A.C.T.	N,R	‡
	Vic.	N,R	‡
	A.C.T.	N,R	Burdon & Chilvers (1974)
E. (*M.*) *globoidea* Blakely	Vic.	P,C	‡
E. (*S.*) *globulus* Labill.	S. Africa		Petrak & Sydow (1924)
	Hawaii	P,C	Hodges (pers. comm.)
	Vic.	P,R	‡
	Tas.	P,C	‡
E. (*S.*) *globulus* Labill. subsp. *bicostata* (Maid. *et al.*) Kirkpatrick	Vic.	P,C	‡
E. (*S.*) *globulus* Labill. subsp. *globulus* Kirkpatrick	Vic.	P,C	‡
E. (*S.*) *grandis* Hill ex Maid.	Vic.	P	‡
E. (*M.*) *gregsoniana* L. Johnson & D. Blaxell	A.C.T.	O,R	‡

Table 1. *Recorded hosts of* A. eucalypti

Host	Location	Status*	Reference
E. (*S.*) *johnstonii* Maid.	Tas.	N,R	‡
E. (*S.*) *lehmannii* (Schau.) Benth.	Vic.	O,C	‡
E. (*M.*) *macrorhyncha* F. Muell. ex Benth.	Vic.	N,R	‡
E. (*S.*) *microcorys* F. Muell.	Vic.	P	‡
E. (*M.*) *niphophila* Maid. & Blakely	S. Aust.		Anon. (1959)
E. (*S.*) *nitens* (Deane & Maid.) Maid.	Vic.	N,C	Neumann & Marks (1976)
	A.C.T.	O	‡
E. (*M.*) *nitida* Hook.	Tas.	N,C	‡
	Vic.	P,C	‡
E. (*M.*) *obliqua* L. Herit.	Vic.	N,C	‡
	Tas.	P,N,C	‡
E. (*M.*) *pauciflora* Sieb. ex Spreng.	A.C.T.	N,C	Burdon & Chilvers (1974)
	A.C.T.	N,C	‡
	Vic.	N,C	‡
	Vic.	O	‡
E. (*S.*) *perriniana* F. Muell. ex Rodway	Vic.	O,R	‡
E. (*M.*) *pilularis* Sm.	Vic.	P,C	‡
E. (*M.*) *radiata* Sieb. ex DC.	A.C.T.		Burdon & Chilvers (1974)
	A.C.T.	N	‡
	Vic.	N,R	‡
E. (*M.*) *regnans* F. Muell.	Vic.	N,C	‡
	Tas.	N,C	‡
E. (*S.*) *saligna* Sm.	Hawaii	P,R	Hodges (pers. comm.)
E. (*M.*) *sieberi* L. Johnson	Vic.	P	‡
	A.C.T.	N,R	‡
E. (*M.*) *stellulata* Sieb. ex DC.	S. Aust.		Anon. (1959)
E. (*E.*) *tetragona* (R.Br.) F. Muell.	A.C.T.	O,C	‡
E. (*S.*) *viminalis* Labill.	Tas.	P,R	‡
E. (*S.*) *woodwardii* Maid.	Vic.	O,R	‡
Angophora costata (Gaertn.) Druce	Vic.	P,C	‡

* N, natural forest; P, plantation; O, ornamental; C, common; R, rare.
† Subgenera: *M.*, *Monocalyptus*; *S.*, *Symphyomyrtus*; *E.*, *Eudesmia*.
‡ Recorded in present study.

Culturing

It was difficult to isolate *A. eucalypti* directly from surface-sterilized lesion margins. As the fungus grew very slowly, it was invariably overgrown by secondary invaders. Ascospores deposited on Water Agar commonly gave almost 90% germination within 12 h at 20 °C. Germination occurred from either cell, and occasionally from both cells of ascospores (Fig. 19). Ascospores became melanized as germination proceeded and germ tubes were heavily melanized. When ascospores were isolated on to agar media, development of visible colonies took 2 weeks. Two different colony types, both heavily melanized, developed. One type had compact black mycelium and later developed grey fluffy mycelium in the older central part of the colony. The other type consisted entirely of brown to black open fluffy mycelium. Colonies grew at rates of 2·4–5·4 mm per month, regardless of the nutrient medium and the source of ascospores (foliage or leaf litter, *E. obliqua* or *E. regnans*). Sporophores of *A. eucalypti* were formed only by fluffy colony types growing on eucalypt leaf pieces sterilized by gamma-irradiation. Pycnidia were formed after 2–3 months' incubation, followed by pseudothecia 2–3 months later. Pseudothecia were never formed in the absence of pycnidia. Sporophores were formed on both surfaces of leaf pieces sitting flat on the agar medium and had the same physical association with the leaf surface as they did on lesions in the field. Up to 100 pycnidia and 10

Table 2. *Comparison of dimensions of structures of* A. eucalypti *on various hosts*

Fungal structures	*E. regnans* (Narbethong)		*E. regnans* (Beech forest)		*E. baxteri* (Narbethong)		*E. obliqua* (Narbethong)	
	x̄	Range	x̄	Range	x̄	Range	x̄	Range
Ascospore length (μm)	11·6	(8·7–14·1)	11·3	(9·2–12·2)	12·9	(10·7–16·1)	12·9	(10·9–14·0)
Long cell length (μm)	6·2	(4·6–7·1)	5·9	(5·1–6·9)	6·8	(5·6–8·2)	7·3	(5·7–7·8)
Long cell width (μm)	3·7	(3·1–4·7)	3·8	(3·1–4·9)	4·1	(3·1–4·9)	3·4	(2·6–4·2)
Short cell length (μm)	5·4	(4·3–6·3)	5·3	(4·1–6·3)	6·1	(5·1–8·2)	5·5	(4·9–6·2)
Short cell width (μm)	4·4	(3·5–5·5)	4·4	(3·6–5·1)	4·9	(3·9–5·9)	4·5	(3·1–5·2)
Pycnidiospore length (μm)	15·1	(2·0–28·6)	29·6	(20·0–36·0)	33·3	(24·5–38·8)	15·6	(5·0–26·0)
Lesion size (mm)	3·7	(1·5–8·2)	9·4	(2·0–18·4)	6·0	1·9–11·4)	4·5	(1·0–8·4)

Fungal structures	*E. globulus* (Hawaii)		*E. globulus* (Nowa Nowa)		*A. costata* (Nowa Nowa)	
	x̄	Range	x̄	Range	x̄	Range
Ascospore length (μm)	12·4	(10·9–13·0)	12·6	(10·4–13·5)	12·5	(10·4–15·1)
Long cell length (μm)	6·8	(5·7–7·8)	7·4	(5·7–9·9)	6·9	(3·6–8·3)
Long cell width (μm)	3·2	(2·6–4·2)	3·7	(2·8–4·4)	3·8	(3·1–6·2)
Short cell length (μm)	5·2	(5·2–5·5)	5·3	(4·2–5·7)	5·3	(4·8–6·5)
Short cell width (μm)	4·6	(1·4–5·2)	4·6	(4·2–5·2)	4·5	(3·6–6·8)
Pycnidiospore length (μm)	14·1	(9·0–23·0)	24·4	(6·0–37·0)	17·1	(13·0–23·0)
Lesion size (mm)	9·4	(1·6–19·6)	5·0	(1·7–7·8)	4·6	(1·0–11·2)

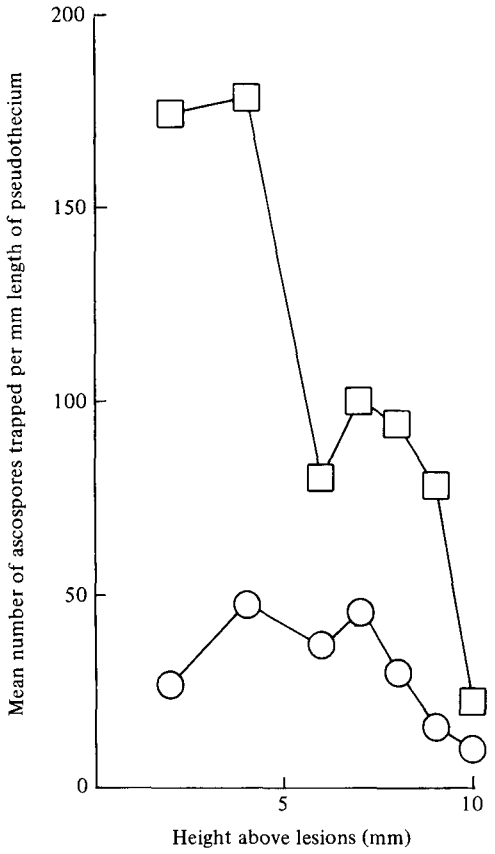


Fig. 17. Ejection of ascospores to various heights above pseudothecia on lesions on leaf litter and foliage over a period of 100 h. □, lesions on foliage; ○, lesions on leaf litter.

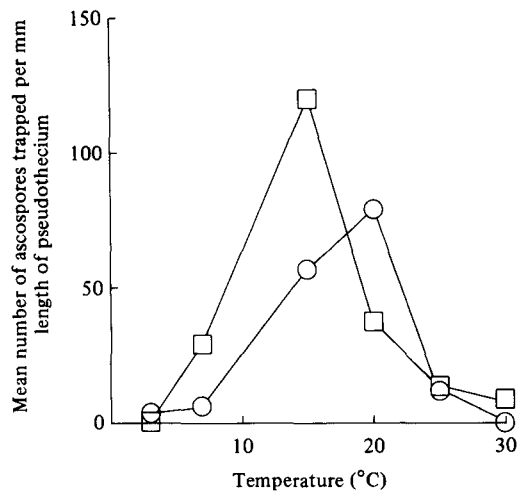


Fig. 18. Release of ascospores from pseudothecia on lesions on leaf litter and foliage at various temperatures. □, lesions on foliage; ○, lesions on leaf litter.

pseudothecia were produced on a 25 × 25 mm piece of leaf in culture. Pseudothecia and ascospores formed in culture were identical to those of *A. eucalypti*, and pycnidia and pycnidiospores were identical to those of *T. eucalyptina* formed on lesions in the field. Pycnidia and pseudothecia were both regularly formed on colonies derived from a single ascospore.

Pycnidiospores (Fig. 7) were never observed to germinate on agar media.

Sporulation

Ascospores were forcibly ejected up to a height of 10 mm above lesions of leaf litter and foliage (Fig. 17). In general many more spores were released from foliar lesions than from lesions on leaf litter. Ascospores were not released from pseudothecia exposed to relative humidities below 98%. At the very high humidities at which ascospores were released it appeared that pseudothecia had become moist. A further experiment showed that in general ascospores were released only after pseudothecia had been wetted (Tables 3, 4). A wetting period as short as 1 min was sufficient to result in ascospore release. Most spores were released within the first 3 h after wetting of lesions. Spore release was delayed somewhat in lesions wet for only short periods: if lesions were soaked from 1–15 min, it appeared that the asci required a period of 1–2 h in the moist Petri dishes for further maturation. Spore release was prolific when infected leaves were soaked in distilled water for 1 h and then placed in vertical racks inside a spore trap through which a stream of air was drawn. Over a period of about 1 h the leaves slowly dried and ascospores were released, to be collected by impaction on coverslips placed below holes in the base of the trap.

Initial observations in the field and on material collected for sporulation tests in the laboratory provided evidence that sporulation varies with season, being least in winter and greatest after rains in spring, summer and autumn.

Ascospores were released over the temperature range 3–30°, with optima at 15–20° (Fig. 18). Many more ascospores were released during light treatments than in the dark, whether the treatment was given before or after wetting of lesions (Table 4). Spore release was greatest when lesions were exposed to light both before and after wetting.

Table 3. *Period of lesion wetness required to promote ascospore release*

Soaking time (min)	Spore-release at intervals after soaking (h)									
	0–0·25*	0·25–0·3*	0·3–1·0*	1·0–2·0	2·0–3·0	3·0–4·0	4·0–5·0	5·0–6·0	6·0–7·0	7·0–21·5
0	0	0	2·5	0	0	0	0	0	0	0
1	72	4	8	189	248	89	60	8	21	13
5	124	7	22	35	36	16	9	11	30	2
15	304	49	55	143	160	47	40	11	14	3
30	239	127	65	8	3	0	5	12	17	13
60	429	35	2	13	16	11	0	4	6	0
120	153	17	7	61	17	18	1	21	36	2

* Means of 2 expts.

Table 4. *Light, humidity and lesion wetness required to promote ascospore release from lesions*

Light treatment		Ascospore release			
		Lesions wetted		Lesions not wetted	
Before wetting	After wetting	Humid	Dry	Humid	Dry
L	L	399^{7^4}	67^2	4^2	0^2
L	D	179^{7^2}	—	—	—
D	L	102^{2^2}	—	—	—
D	D	257^4	0^2	239^4	0^2

L, Light; D, Dark; superscripts, no. of replicates; —, not tested.
Before wetting light treatment = 12 h; wetting period = 30 min.

Pathogenesis

After leaves had been inoculated in Petri dish moist chambers with ascospores shed from lesions attached to the lids, germination of ascospores on the leaves was observed. Four months after inoculation many small lesions, up to 3·0 mm diam and developing only part-way through leaves, were observed on inoculated parts of leaves (Fig. 20). At this stage pycnidia were present on the lesions. Leaves that developed lesions had been partly expanded and 15–40 mm long, with a soft cuticle, at the time of inoculation. Inoculation of fully expanded leaves did not induce disease.

Successful inoculation was achieved by clipping to unhardened leaves plastic blisters containing a sporulating lesion. Ascospores germinated 12 h after being deposited, producing heavily melanized, septate, branched germ-tubes. On germination, ascospore walls also became melanized. Within 2–11 d, appressoria were formed as a result of swelling and wall-thickening of a germ-tube cell or as a single-celled outgrowth from a germ-tube cell or ascospore. Commonly 1–2(–3) appressoria formed on germ-tubes originating from a single ascospore (Fig. 21). Appressoria were formed on both soft young leaves and fully expanded, hardened older leaves but no further development occurred on the latter. After 15 d on some leaves, particularly those with a dense deposition of ascospores, anastomosis occurred between germ-

Figs 19–26. *Aulographina eucalypti*

Fig. 19. Germination of ascospores on Water Agar. Bar = 100 μm.

Fig. 20. Small leaf spots developing on *E. obliqua* 4 months after inoculation with ascospores of *A. eucalypti*. Bar = 10 mm.

Fig. 21. Ascospore (arrowed) germinating on leaf of *E. obliqua*, forming two melanized germ-tubes, each of which has formed a melanized appressorium. Bar = 10 μm.

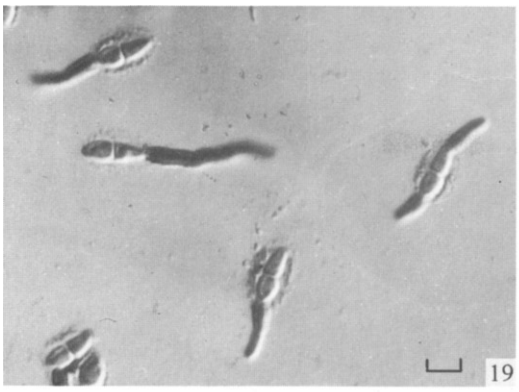
Fig. 22. Anastomosis (arrowed) between germ-tubes. Bar = 10 μm.

Fig. 23. Micrograph of penetration of leaf of *E. obliqua* from appressorium of *A. eucalypti*, showing initial sub-cuticular hyphae (arrowed). Bar = 10 μm.

Fig. 24. Micrograph of penetration of leaf of *E. obliqua* 19 days after inoculation, showing branched sub-cuticular hyphae (arrowed). Bar = 10 μm.

Fig. 25. Leaf of *E. obliqua* infected with *A. eucalypti*. Bar = 10 mm.

Fig. 26. Autoradiograph of leaf shown in Fig. 25 after uptake of $^{14}CO_2$. Bar = 10 mm.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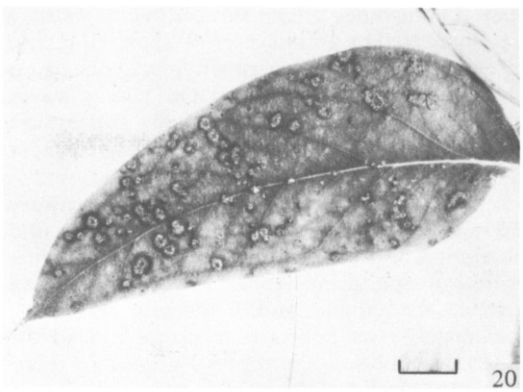
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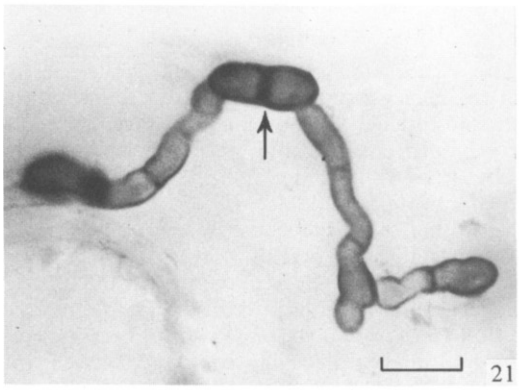
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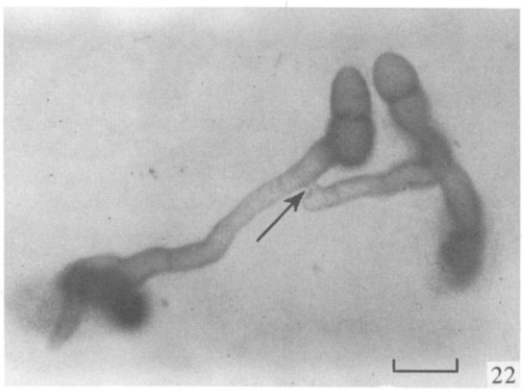
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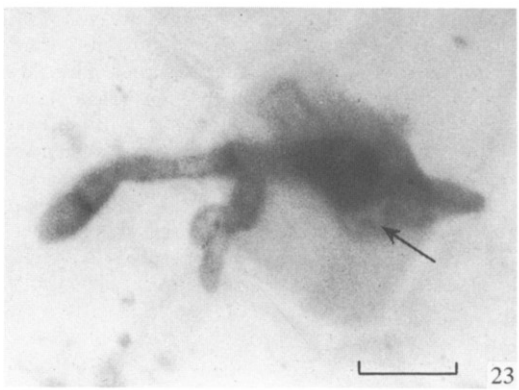
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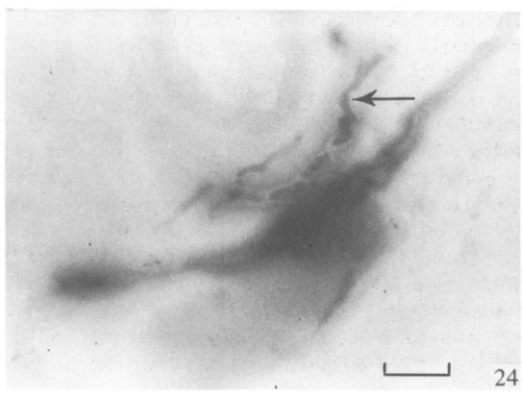
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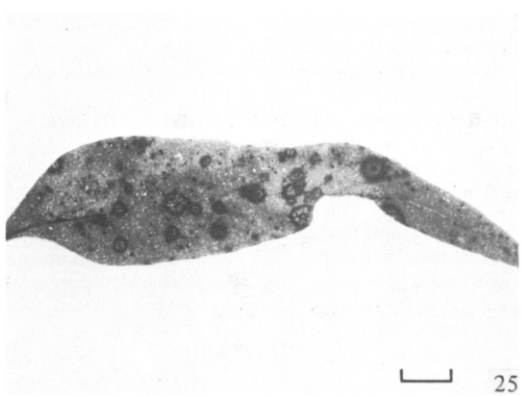
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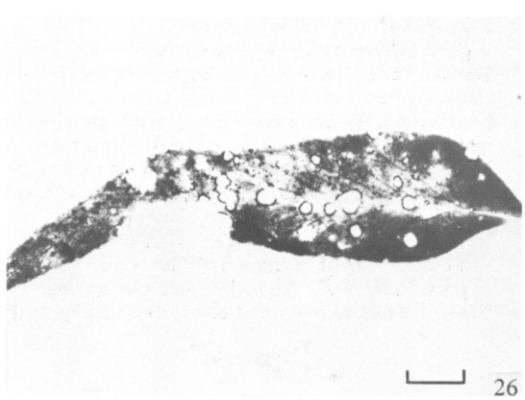
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tubes. Germ-tubes either fused directly or they were connected by a short, narrow hypha (Fig. 22). On occasions, a short, narrow hypha connected a germ-tube and an ascospore. On young leaves anastomosing germ-tubes formed appressoria and penetration structures in the same way as non-anastomosing germ-tubes.

Leaves stained by the PAS method always developed a red zone in the cuticle around appressoria. On young leaves below each appressorium, a spherical hyphal knob was formed between the cuticle and epidermis (Fig. 23). Immediately after penetration, protoplasm of the epidermal cell below the appressorium stained red with PAS. At later stages of penetration similar staining occurred in adjacent epidermal cells. Staining of sections with lactophenol cotton blue revealed that these cells were necrotic. Frequently all appressoria formed by the germ-tube of a single ascospore produced penetration pegs. Between 15 and 25 d after inoculation penetration pegs gave rise to thick-walled, branched hyphae in the sub-cuticular region (Fig. 24). By 34 d after inoculation sub-cuticular hyphae were longer and had developed septa. By 64 d after inoculation round necrotic spots, less than 1 mm diam and penetrating only part-way through the leaf, were visible. By this time germinated ascospores with germ-tubes and appressoria had, in most cases, been broken down or dislodged and only penetration points were visible. At this stage extensively-branched septate hyphae were seen to have grown radially from the original site of penetration and had formed a regular stromatic layer. The hyphae still appeared to be mainly located between the cuticle and epidermis. Protoplasm of epidermal cells near the point of penetration was now darkly stained by the PAS method and had collapsed. Subcuticular hyphae were seen just beyond the stained epidermal cells.

Penetration and growth of subcuticular hyphae were similar on both adaxial and abaxial surfaces of leaves, although by 25–34 d after inoculation growth of sub-cuticular hyphae was more extensive on the abaxial than on the adaxial surface. In all inoculations there was great variability in germination rate and percentage, germ-tube growth rate, and growth rate of subcuticular hyphae.

Germ-tubes occasionally grew into stomatal pores but hyphae were never observed to penetrate beyond the pore. Penetration occasionally occurred through the cuticle above guard cells, giving rise to subcuticular hyphae.

Ascospore suspensions ($1{\cdot}4 \times 10^5$ and $2{\cdot}4 \times 10^5$ ascospores ml^{-1}) have been successfully used for inducing disease. These ascospores had begun to germinate prior to inoculation. The number of lesions forming per unit leaf declined with increasing leaf age. Fourteen weeks after inoculation, lesions were 0·5–3 mm diam and some had developed pycnidia. Lesions were slightly raised above the leaf surface and some of the surrounding cuticle was cracked. Stromatic hyphae were evident between the cuticle and epidermis just beyond the central necrotic area when lesions were stained with PAS. Melanized surface hyphae were sparse and rarely extended to the margins of the stroma. Surface hyphae on slightly larger lesions appeared to emerge from the stomata or cuticle above stromatic hyphae. The extent of lesion development was variable: some penetrating hyphae had not developed into lesions after 64 d.

Physiological studies

The anthocyanin pigment formed around lesions in the cooler months of May to October was extracted and identified by comparison with a known compound using thin-layer chromatography. The R_F value was the same as that of cyanidin-3-glucoside.

Autoradiography of leaves exposed to $^{14}CO_2$ revealed that in healthy leaves the radioactive isotope was evenly distributed while in diseased leaves it had accumulated in the purple margins around lesions and was absent from necrotic areas except for a slight accumulation in concentric ring on each lesion roughly corresponding with a ring of sporophores. This slight accumulation is visible on the original autoradiographs but not on the prints made from them (Figs 25, 26). The mean concentration of unhydrolysed sugars in glucose equivalents was $1{\cdot}19 \pm 0{\cdot}32$ mg g^{-1} in diseased tissue and lesion margins compared with $0{\cdot}62 \pm 0{\cdot}57$ mg g^{-1} in healthy tissue.

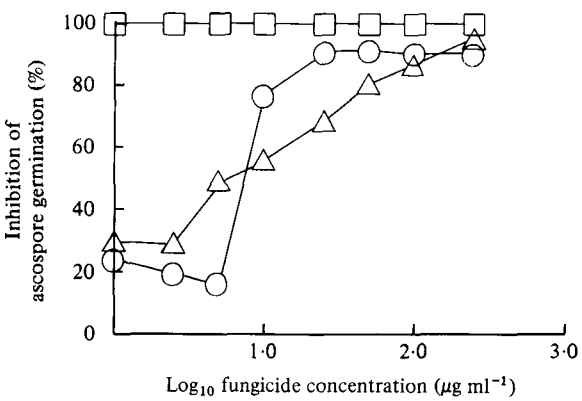

Fig. 27. Inhibition of ascospore germination by various fungicides (germination was defined as the formation of a germ-tube at least half as long as the ascospore). □, Captan; △, Benlate; ○, Copper oxychloride.

Captan was the most effective fungicide in inhibiting ascospore germination of *A. eucalypti*, giving complete inhibition at a concentration as low as 1 μg ml^{-1} (Fig. 27).

DISCUSSION

The circular leaf spots induced on eucalypts by *A. eucalypti* are easily distinguished by development of necrotic tissue only part-way through the leaf and by the presence of distinctive pimple-like pycnidia and elongate, occasionally branched pseudothecia on the lesion surface. Cultures of the fungus established on sterilized eucalypt leaf pieces from single ascospores formed both pycnidia and pseudothecia, confirming that the pycnidial fungus originally described by Petrak & Sydow (1924) as *Thyrinula eucalyptina* is in fact the conidial state of *A. eucalypti*. Development of pseudothecia in single-ascospore cultures indicates that *A. eucalypti* is homothallic. The role of the conidial state is unknown: pycnidiospores contain very little cytoplasm and they have not been observed to germinate on a variety of agar media. In cultures established from ascospores and on lesions on leaves, pycnidia always developed before pseudothecia, providing evidence that the pycnidiospores may function as male gametes rather than as propagules. Hansford (1954) considered that the main point of difference between *Lembosiopsis eucalyptina* and *L. australiensis* was the absence of external mycelium in the latter. As the external mycelium is often sparse (Müller & von Arx, 1962) and often disintegrates on older lesions, this does not seem to be sufficient to distinguish the species. Müller & von Arx (1962) observed a 'subcuticular hypostroma' composed of reddish-brown, thick-walled hyphae, 4–7 μm broad, spread out between the epidermal cells, and emerging abundantly through the cuticle beneath sporophores and elsewhere. Hansford (1954) also observed emergence of hyphae through the cuticle with *L. australiensis* and this was commonly observed in the present study. Dimensions of asci and ascospores reported for *L. australiensis* are similar to those of *A. eucalypti* as given by Müller & von Arx (1962) and as found in the present study on a range of eucalypt species. There seems no doubt that *L. australiensis* is a synonym of *A. eucalypti*, as suggested by Müller & von Arx (1962).

Lesions developed very slowly and for this reason were most evident on older leaves. West (1980) reported that lesions caused by *A. eucalypti* were considerably larger and more frequent on *E. obliqua* trees infected with the root-rotting pathogen *Phytophthora cinnamomi* Rands than on nearby uninfected trees of the same species. It is possible that the leaves remained longer on the root-rot affected trees, thus allowing greater development of *A. eucalypti*. Alternatively, leaves of root-rot affected trees may have been more susceptible to infection by *A. eucalypti*, or the microclimate around them may have been more favourable for infection. The slow invasion of leaves by the fungus allows ample time for development of a cork cambium around the lesions. While initial subcuticular invasion of leaves by the fungus appeared to adversely affect adjacent epidermal cells, as indicated by PAS staining, it is possible that many of the cells in the lesion are killed as a result of being isolated by the cork barrier. It appears that the cork barrier does not completely restrict the lateral growth of the fungus: melanized surface hyphae and sub-cuticular hyphae were commonly found growing beyond the edges of the cork cambium and the necrotic spot. Heather (1961) reported that cork barriers are commonly formed in eucalypt leaves in response to fungal invasion. He identified two types of barrier, one that extends from the adaxial to the abaxial leaf surface, completely surrounding the lesion, and one that forms a saucer-shaped zone around and under the lesion as in the present case.

Stages of fungal invasion have been described from inoculated leaves and from lesions on leaves collected in the field. Leaf invasion by *A. eucalypti* is similar to invasion of apple leaves by *Venturia inaequalis* (Cke) Wint. (Nusbaum & Keitt, 1938) except that with *A. eucalypti* the process is much slower. Both fungi penetrate the cuticle directly from appressoria and form a sub-cuticular stroma. *A. eucalypti* differs from *V. inaequalis* in forming extensive melanized surface hyphae which also colonize the leaf, and in inducing more extensive necrosis of underlying leaf tissue. In *A. eucalypti* sub-cuticular hyphae emerge on to the leaf surface either through stomata or directly through the cuticle while in *V. inaequalis* conidiophores emerge through the cuticle. *A. eucalypti* causes premature defoliation of heavily infected leaves or leaves with infected petioles. With the fall of diseased leaves it is quite apparent that *A. eucalypti* is not rapidly overgrown by saprophytes. It continues to sporulate prolifically for several months after leaf abscission. Leaf litter is certainly a potentially important inoculum source. *A. eucalypti* also forms lesions on and survives for a considerable time on fruits and smooth bark of some *Eucalyptus* species and these lesions are also likely to be important inoculum sources. Epidemic development is likely to be limited by availability of inoculum, which decreases during winter when temperatures fall well below the optimum for sporulation of 15–20° (Fig. 18), by lack of soft young leaves for infection during

winter, and also by lack of free water for spore discharge and infection on hot, dry summer days.

A. eucalypti is similar to *V. inaequalis* (Brook, 1969) in releasing ascospores mostly when wetting of ascocarps occurs in the light rather than in darkness. It follows that, as with *V. inaequalis*, ascospore release by *A. eucalypti* is likely to be more strongly associated with daytime rains than with night rains or dew.

In this study lesions caused by *A. eucalypti* have been found on a wide range of species from both major subgenera of *Eucalyptus*. This appears to contradict the observations of Heather (1971) and Burdon & Chilvers (1974) that *A. eucalypti* tends to occur mainly on species of eucalypts in the subgenus *Monocalyptus*. Burdon & Chilvers (1974) found that, in stands of codominant *Eucalyptus* at four different sites in the Brindabella Mountains, A.C.T., the *Monocalyptus* species *E. radiata*, *E. dives*, *E. delegatensis* and *E. pauciflora* were infected with *A. eucalypti* while the codominant *Symphyomyrtus* species *E. viminalis* and *E. dalrympleana* were not. It seems that in a particular habitat the fungus occurs mainly on one subgenus, while if collections are made from many localities including plantations and gardens, it can be found on both major subgenera of *Eucalyptus* and even on another genus such as *Angophora*. It is significant that one of the reported epidemics of *A. eucalypti* occurred on *E. nitens*, a *Symphyomyrtus* species (Neumann & Marks, 1976). So far there is no evidence of racial differences between the *A. eucalypti* isolates from different eucalypt species. In future work pathogenicity tests will be used to study host specificity of the fungus.

There is no doubt that *A. eucalypti* has an effect on leaf tissue beyond the necrotic spot with which it is associated. Autoradiography of diseased leaves exposed to $^{14}CO_2$ indicated that ^{14}C accumulated in the purple tissue at the margin of the necrotic spot and also in sporophores on the lesion. Extraction of unhydrolysed sugars from lesions, including the purple margins, also provided evidence for accumulation of sugars in the lesion or lesion margin. Meyer & Anderson (1968) reported that an increase in sugar concentration in plant tissue can be associated with accumulation of anthocyanin pigments in the tissues. In the colder months there was marked accumulation of the anthocyanin, cyanidin-3-glucoside, around lesions caused by *A. eucalypti*. It appears that this was associated with sugar accumulation in the lesion margins. There is thus strong evidence that *A. eucalypti*, like many biotrophic plant parasites, acts as a sink for photosynthate in infected leaves although the cork cambium formed around the lesion may also have acted as a nutrient sink.

There is no doubt that *A. eucalypti*, although causing necrosis of leaf tissue, is a highly adapted leaf pathogen, with many attributes of a biotrophic parasite. It actively invades healthy, expanding leaves, has a very specific mode of penetration and colonisation, survives for extended periods on leaves, and grows very slowly and sporulates with difficulty in culture.

The authors gratefully acknowledge the invaluable assistance of Dr G. C. Marks, Forests Commission, Victoria. This work was supported financially by the Forests Commission Victoria and by a Rural Credits Development Fund Grant from the Reserve Bank of Australia.

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(*Received for publication 27 January 1983*)