ASSESSMENT OF PLATYPUS SUBGRANOSUS AS A VECTOR OF CHALARA AUSTRALIS, CAUSAL AGENT OF A VASCULAR DISEASE OF NOTHOFAGUS CUNNINGHAMII

G. A. KILE and M. F. HALL
CSIRO Division of Forestry and Forest Products, Stowell Avenue, Battery Point, Tasmania 7004, Australia

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ABSTRACT

The potential role in Australia of **Platypus subgranosus** Schedl (Coleoptera: Curculionidae: Platypodinae) as a vector of the fungus **Chalara australis** Walker & Kile, which causes a vascular disease of **Nothofagus cunninghamii** (Hook.) Oerst. was studied by determining the way in which the beetle carries its mycoflora, by making direct isolations from living beetles, and by induction of beetle attack on disease-free trees and billets of **N. cunninghamii**. The origin of subsequent **C. australis** infections in these trees or billets was investigated. The relationship between the fungus and the life cycle of the beetle in naturally attacked host trees was also examined.

Chalara australis was isolated from 0.6% of 2966 **P. subgranosus** adults and 1.2% of tunnel walls in billets were infected. Isolations from beetle frass indicated that infection of trees occurred prior to beetle attack. Because saprophytic survival of the fungus in the wood of the dead host trees is limited, there is little opportunity for the fungus to contaminate emerging brood. While a small number of beetles may possibly carry and transmit **C. australis**, the fungus is not dependent on the beetle for dissemination or ingression.

Keywords: disease transmission; **Platypus subgranosus**; **Nothofagus cunninghamii**; **Chalara australis.**

INTRODUCTION

Nothofagus cunninghamii (myrtle), the major tree species of the cool temperate rainforest of Tasmania and the southern highlands of Victoria, is affected by widespread and locally severe disease (Elliott *et al.* 1987; Kile & Walker 1987). Superficially there is a close association between attack of the stems of *N. cunninghamii* trees by the xylomycetophagous beetle *Platypus subgranosus* and the development in their crowns of characteristic wilt symptoms. Howard (1973) hypothesised that the death of *N. cunninghamii* was caused by a pathogenic fungus transmitted by this borer. Kile & Walker (1987) established the veracity of the first element of Howard's hypothesis by fulfilling Koch's postulates for *Chalara australis,* a hyphomycete they consistently isolated from the discoloured outer wood in the roots and lower stems of diseased trees.

The survey of Elliott *et al.* (1987) confirmed that virtually all *N. cunninghamii* which die in Tasmanian rainforests are attacked by *P. subgranosus*. The clumping of diseased trees shown by that survey together with the initial observations of Kile & Walker (1987) on the apparent relationship between *P. subgranosus* attack and *C. australis* infection suggested three hypotheses which alone, or in combination, could explain dispersal and infection by *C. australis* – (a) direct vectoring by *P. subgranosus* (or unknown insect(s)), (b) airborne or waterborne infection of wounds with below-ground spread by rootgraft or root contact (or unknown below-ground vectors), or (c) random attacks by *P. subgranosus* on healthy trees or on trees rendered susceptible by stress factors, followed by infection of the wood via the borer tunnels, through airborne, insect-dispersed, or waterborne inoculum of *C. australis*. A knowledge of the mode of fungal dispersal is critical to an understanding of the epidemiology of this important disease and for developing disease limitation strategies in *N. cunninghamii* rainforest disturbed by human activity. This paper reports studies which assessed the role of *P. subgranosus* as a possible vector of *C. australis*.

MATERIALS AND METHODS

Carriage of Fungal Propagules by *P. subgranosus*

Platypus subgranosus is xylomycetophagous (Webb 1945; Hogan 1948) and several species of suspected ambrosial fungi have been isolated consistently from male and female beetles (Webb 1945; Kile & Walker unpubl. data). Many ambrosia beetles disseminate their symbiotic fungi in special organs of ectodermal origin called mycetangia (Batra 1963; Francke-Grossmann 1967). To determine if suspected ambrosial fungi are carried in mycetangia and how propagules of *C. australis* might be transported if *P. subgranosus* were a vector, beetles were examined for such structures and for fungal spores using light and scanning electron microscopy.

For light microscopy, male and female *P. subgranosus* excavated from tunnels in dead *N. cunninghamii* trees were fixed and embedded in paraffin. Serial transverse, sagittal, and dorso-ventral sections were stained with the modified Gram Weigert stain following the procedures of Leach (1940) and Farris (1963). Fernando (1960) found that this stain selectively identified fungal deposits in the scolytid *Xyleborus fornicatus* Eichhoff.

Tests for the Presence of *C. australis* Propagules on Adult *P. subgranosus*

Direct isolations from living beetles

Male and female beetles were plated on potato dextrose agar (PDA), 3% malt extract agar (ME), and malt extract agar plus 0.5% yeast extract (YEME).

Sources of *P. subgranosus* were:

(i) Newly emerged beetles from infested billets of *N. cunninghamii* (Esperance Valley, Tasmania) incubated in a black polythene tent. Emerging beetles were collected from November 1983 until March 1984 in a translucent plastic bottle attached to the tent. Beetles used for plating were collected over the previous 24-hour period.

(ii) By trapping free-flying *P. subgranosus* in terylene mesh nets on a rotary trap on 10 separate occasions during the 1984/85 and 1985/86 flight seasons. The trap was located in a myrtle wilt infection centre in rainforest near the Little Florentine River (LFR) south-central Tasmania (site 2, Elliott *et al.* 1987).

(iii) By trapping emerging *P. subgranosus* in terylene mesh cages erected around the lower stems of five *C. australis*-infected *N. cunninghamii* brood trees at LFR. Beetles were collected from cages every 2–4 days during the latter part of the 1985/86 flight season.

Prior to plating, some of the beetles collected from tented billets were surface-sterilised in 1% sodium hypochlorite for 5–10 minutes, while others were treated with the wet-dry fractionation technique of Francke-Grossmann (1956) as modified by Batra (1963), to germinate and kill external fungal spores and to promote the isolation of any mycetangial fungi. Beetles from sources (ii) and (iii) were not pretreated before plating because no additional fungi were consistently isolated after pretreatment. For controls the dorsal surface of some beetles was touched on a culture plate of *C. australis* 1–2 hours before plating the beetles.

Inoculation of whole or macerated P. subgranosus *beetles into seedlings or stem discs of* N. cunninghamii

Platypus subgranosus obtained from tented billets were macerated in sterile distilled water or washed by gently agitating in a Sorvall Omni Mixer and the resulting suspension was inoculated into *N. cunninghamii* seedlings (Kile & Walker 1987). After inoculation, seedlings which died were dissected and isolations were made for *C. australis;* after 6 months all remaining living seedlings were dissected and isolations made from them. *Platypus subgranosus* from (ii) and (iii) were macerated and dispersed on to freshly cut stem discs (5 cm long × 4–6 cm diameter) in polypropylene dishes sealed with a lid containing a bouchon. Other discs were inoculated by placing a single beetle in contact with the distal end of a 6-mm-wide and 1-cm-deep hole drilled into the top of each disc. Discs were incubated for 3–4 weeks at 20°C, the optimum temperature for growth of *C. australis,* and moistened weekly with sterile water. Controls comprised seedlings and discs inoculated with sterile distilled water, and with intact beetles or macerates or washings of beetles deliberately contaminated with *C. australis.* The latter were included to test the effectiveness of the method used in removing any *C. australis* conidia from the beetle cuticle, and the possible toxic effects of the body contents of *P. subgranosus* on the viability of *C. australis* conidia. Beetles were contaminated by allowing them to crawl on *C. australis* plates for 30 minutes prior to washing or maceration and inoculation.

Induced P. subgranosus *attack on trees and billets of* N. cunninghamii *and its relationship to* C. australis *infection*

As summer wounding of trees induces *P. subgranosus* attack (H. J. Elliott pers. comm.; Kile & Walker 1987), 36 healthy *N. cunninghamii* trees were wounded in January 1985 to study the relationship between attack and *C. australis* infection. Single wounds were made on each tree by removing a panel of bark approximately half the

tree circumference wide × 0.8 m high, starting from 0.5 m above ground-level. The lower stem wound zones of 12 control trees were wrapped in hessian to exclude *P. subgranosus,* and the wounds on the other 24 trees were left exposed. To prevent *C. australis* infection during wounding, the area of bark to be removed was flamed with 95% ethanol prior to removal with a sterile hatchet. Records of the number of *P. subgranosus* tunnels initiated on trees were kept and trees were felled after 9 weeks to check for *C. australis* infection. Because billets from freshly felled trees of *N. cunninghamii* are susceptible to *P. subgranosus* attack during its flight season and may become infected by *C. australis,* three series of billets were exposed in the forest during the 1984/85 and 1985/86 flight seasons and the winter/early-summer of 1986.

(i) Series I 1984/85.

Billets (1.7 m length and 0.2 m mean diameter) were cut from healthy *N. cunninghamii* trees at three rainforest sites (Esperance (E), Arve (A) 1 and 2) in southern Tasmania. To prevent *C. australis* infection during preparation, the bark of cutting zones was flamed with 95% ethanol, the chainsaw blade was washed with ethanol, and the ends of billets were coated with bituminous mastic immediately after cutting. Control billets were enclosed in hessian bags to prevent *P. subgranosus* attack.

(ii) Series II 1985/86.

Billets (1.0 m length and 0.2 m mean diameter) were exposed at Sites E and LFR in December 1985 and in January and February 1986. Preparation of billets was similar to that in Series I except more rigorous precautions were taken to prevent *C. australis* infection other than via *P. subgranosus* tunnels. After cutting, billet ends were flamed with 95% ethanol and heated with an LP gas flame prior to the application of an impermeable silicon sealant. After sealing, some billets were treated to render them more susceptible to *P. subgranosus* attack either by immersing the billets in water for 48 hours or by scorching a 0.1-m^2 panel of bark in the middle of the billet (Elliott *et al.* 1983). Other billets were left untreated. At both sites billets were divided into two groups, one exposed in the open on a rack 0.6 m above ground-level, and the other on a similar rack beneath a corrugated iron shelter to prevent wetting of the billets by rain.

(iii) Series III winter/early-summer 1986.

To explore further the likely mode of entry of *C. australis* into billets, additional billets of similar size, and prepared in the same manner as those in Series II, were exposed at Site LFR during the non-flight season of *P. subgranosus.* Groups of four billets were racked inside and outside the shelter in July, August, October, and December 1986. Within each group, two of the billets had a panel of 30 holes (each 2 mm diameter × 3 cm depth) drilled 3 cm apart mid-billet to study the entry of *C. australis* through simulated *P. subgranosus* wounds. One each of the drilled and untreated billets was enclosed in terylene mesh bags to prevent access by *P. subgranosus* or other insects.

For each series the number of *P. subgranosus* tunnels was recorded at regular intervals and the billets were dissected after 7–10 weeks.

Relationship Between the Life Cycle of P. subgranosus, Infestation by the Beetle, and Infection of the Host by C. australis

Isolation of C. australis *from* P. subgranosus *frass on trees and billets of* N. cunninghamii *LFR site*

Frass from individual *P. subgranosus* tunnels in 29 naturally attacked trees was plated on 3% ME during February-March 1986. The trees were in six disease categories – namely, healthy with current attack, early symptoms, dead >70% foliage retained, dead 20–70% foliage retained, dead <20% foliage retained, and dead with fine twigs only. Frass was also plated from three trees inoculated with *C. australis,* from eight trees scorched or ring-barked to induce *P. subgranosus* attack in December 1985, and from Series II billets. Piles or cylinders of fresh frass protruding from tunnels, or adhering to the bark, were removed with sterile tweezers. The plates were incubated at 20°C for 4–7 days and assessed for *C. australis.* Adult frass (fine splinter-like wood fragments) and larval frass (small rounded wood particles) were plated separately.

Potential for within-host contamination by C. australis *of pre-emergent* P. subgranosus

The sporulation of *C. australis* in *P. subgranosus* tunnels, and hence the potential for fungal contamination of pre-emergent adult beetles, was examined by obtaining from each of three naturally attacked trees in each of the six disease categories specified in (a), a single disc of beetle-infested wood (5–7 cm thick by 35–70 cm diameter) at 90–120 cm above ground-level. The discs were split to expose tunnel walls in the outer wood (arbitrarily defined as the 3-cm cylinder of wood nearest the cambium) and the inner wood. The presence of phialophores of *C. australis* in the tunnels was checked by examining scrapings from 1–3 cm length of tunnel wall under a light microscope. Isolations for *C. australis* were made on 3% ME from similar scrapings. Tunnels were categorised as having dark, moderately dark, or light walls (features that are indicative of the age and length of occupancy of the tunnels by beetles and larvae) and whether or not they occurred in wood discoloured by *C. australis.* Thirty tunnels (10 of each wall type) were sampled per disc whenever possible. Phialophores were characterised as non-viable (brown encrusted walls and lacking conidia) or viable (producing conidia). Beetles and larvae obtained from tunnels during dissection of discs were also plated. To assess the survival of the fungus in the stem wood, 25 separate isolation attempts were made from discoloured tissue in both the outer and inner wood zones of each disc.

STATISTICAL ANALYSIS

Data were analysed using generalised linear models (McCullagh & Nelder 1983). The models used for data presented were (1) a linear logistic model as the response variable (attack class) was ordered and (2) binomial error type models with up to four classifying factors (health category, tunnel type, etc.)

RESULTS

Carriage of Fungal Propagules by *P. subgranosus*

Mycetangia did not occur on any part of the beetle cuticle that could be examined by SEM. Both male and female *P. subgranosus* lacked the marked pronotal mycetangial pits evident in other *Platypus* spp. (Roche & Lhoste 1960; Farris & Funk 1965; Francke-Grossmann 1966; Nakashima 1975), the pits in that area being indistinct and superficial (Fig. 1). Fungal spores were present on the cuticles of all beetles examined but especially abundant on anterior dorsal surfaces and in the grooves between body

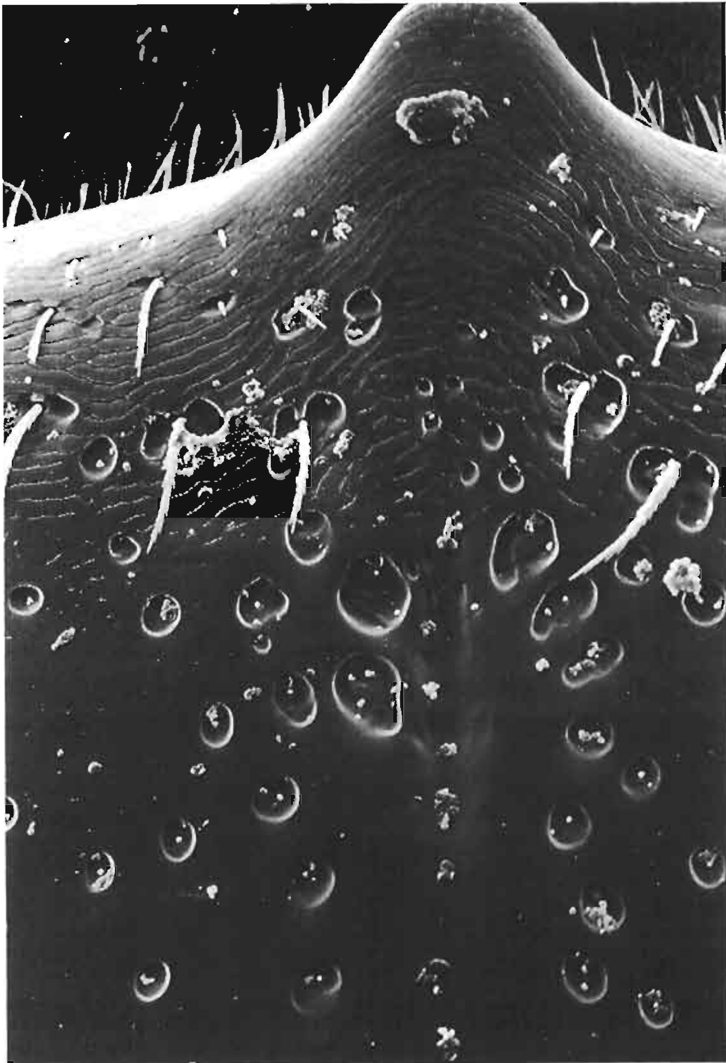

FIG. 1—Superficial pronotal pits of **Platypus subgranosus** (SEM, × 120).

segments (Fig. 2). Sectioning of male and female beetles confirmed the superficial nature of the pronotal pits. No structural modifications or regular fungal deposits were evident in other potential mycetangial areas such as coxal cavities, integumentary folds, and the buccal cavity.

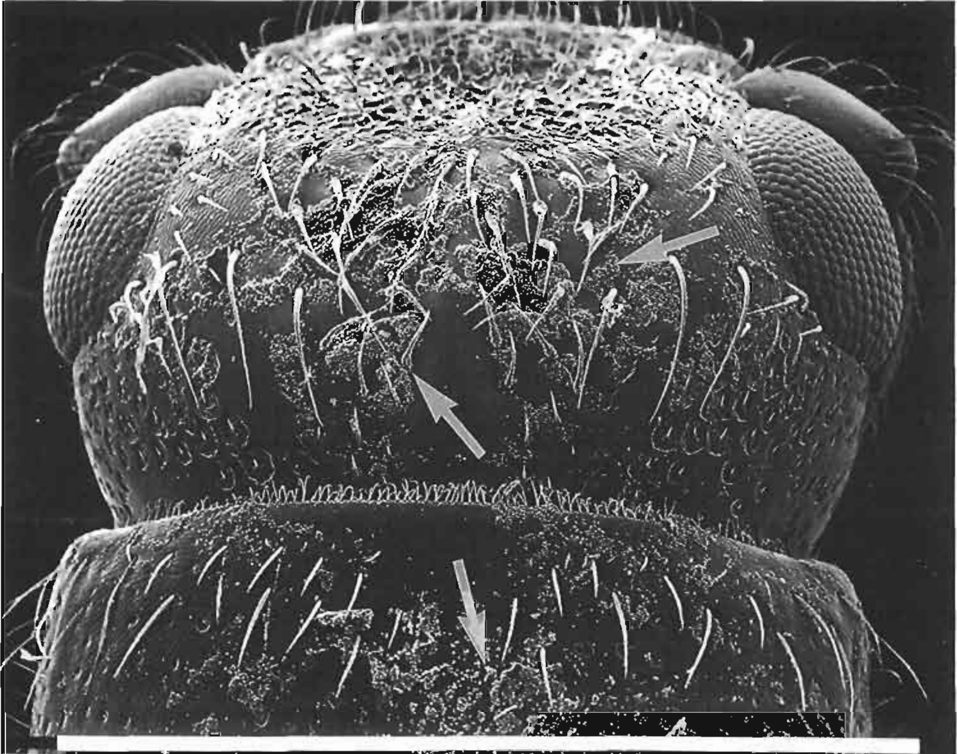

FIG. 2—Fungal propagules (arrowed) on anterior dorsal surfaces of **P. subgranosus** (SEM, Bar = 1 mm).

An unidentified structure occurred singly, or in groups, on the foreheads of both male and female *P. subgranosus*. Large numbers of these structures also occurred in some tunnel wall scrapings from naturally diseased *N. cunninghamii* (Fig. 3). In each structure a curved circular rim of diameter 20–30μm surrounded an inflated centre. Both single and double units of the structure were observed.

Direct Isolations from Living P. subgranosus

Chalara australis was not isolated from any of the 2435 male or female *P. subgranosus* from the three sources (Table 1). However, *C. australis* was reisolated from all deliberately contamined beetles on each media.

Several fungal species were recovered from beetles although the isolation frequency of different fungi varied with source, and with different media and pretreatment of the beetles. *Hormoascus platypodis* (Baker & Kreger-van Rij) V.Arx and *Raffaelea* sp.

were recovered consistently, and *Hyalorhinocladiella* sp., *Pichea acaciae* v.d. Walt, *Ceratocystis piceae* (Munch) Bakshi, *C. moniliformis* (Hedgc.) C. Moreau, *C. pilifera* (Fries) C. Moreau, *Penicillium* spp. (including *P. thomii* Maire), *Mucor* spp., *Trichoderma* sp., and a sterile fungus were also isolated. Occasionally *Aposphaeria* sp., *Cylindrocarpon* sp., *Nodulisporium* sp., and other unidentified fungal and bacterial species were detected.

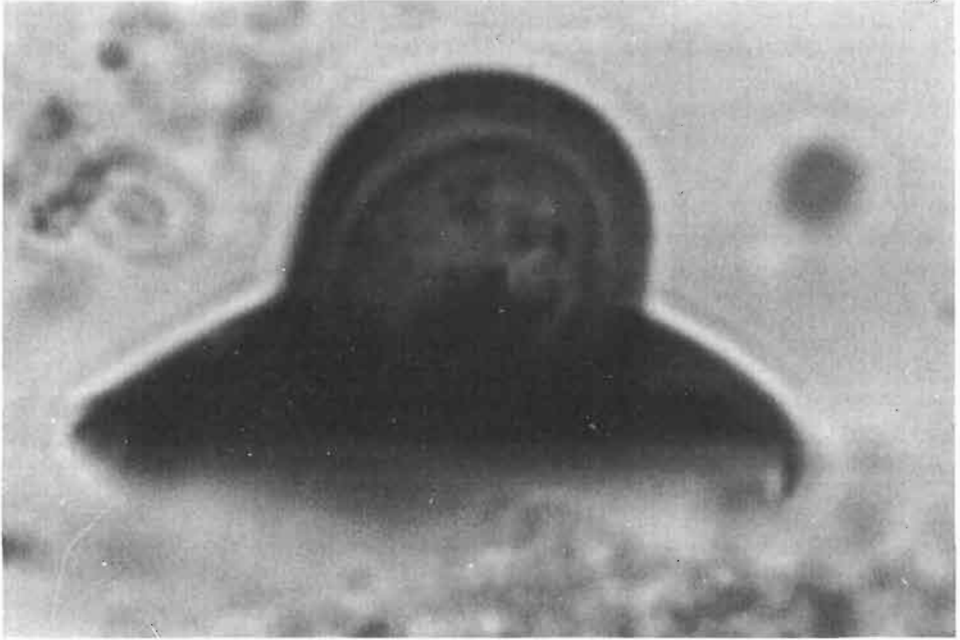

FIG. 3—Unidentified structure found on adult **P. subgranosus** and in tunnel scrapings (× 1000).

TABLE 1—Source and numbers of *Platypus subgranosus* used for direct plating and inoculation studies

P. subgranosus source		No. beetles plated on agar media		No. beetles inoculated into seedlings or on to stem discs	
		Female	Male	Female	Male
1. Tented billets from brood trees	Direct plated	500	500	120(80)*	160(80)
1983–84	Wet/dry fractionation or surface sterilised	561	665		
2. Trapped in flight during 1984–85		2	29		
1985–86		16	49	9	38(9)
3. Temporary cages on brood trees 1985–86		52	61	97(14)	107(16)
Total		1131	1304	226(94)	305(105)

* Figures in parentheses indicate number of beetles used as *Chalara australis* controls

Platypus subgranosus from tented logs yielded many isolations of *Mucor* and *Rhizopus* spp., but these were isolated only occasionally from beetles from the other two sources. Wet-dry fractionation or surface sterilisation reduced the frequency of isolation of all fungi but no other fungal species were consistently isolated.

Inoculation of Whole or Macerated *P. subgranosus* Beetles into Seedlings or Stem Discs of *N. cunninghamii*

The macerates and washings of the *P. subgranosus* collected from tented billets (Table 1) did not cause *C. australis* infection when inoculated into seedlings unless they had been deliberately contaminated with the fungus. Also, 204 male and female *P. subgranosus* from brood trees inoculated on to 64 discs on five separate occasions did not result in *C. australis* infection in the wood. A similar result occurred with 47 *P. subgranosus* trapped during flight (39 discs inoculated on three separate occasions) although in one test involving 20 discs inoculated with a macerate of 16 males and three female beetles, three discs became infected by *C. australis*. Therefore, a possible maximum of 3.6% (19/531, Table 1) of all beetles inoculated into seedlings or discs (excluding controls) were carrying viable *C. australis*. All discs inoculated with *C. australis*-contaminated beetles, from either source, developed infection but all discs inoculated with sterile water remained free of infection.

Induced *P. subgranosus* Attack on Trees and Billets of *N. cunninghamii* and its Relationship to *C. australis* Infection

For trees wounded in January 1985 there was no apparent relationship between *P. subgranosus* attack and *C. australis* infection (Table 2). Three trees with no attack were infected while 11 attacked trees remained uninfected after 9 weeks. *Chalara australis* was not isolated in association with tunnels except when they penetrated extensive volumes of wood discoloured by *C. australis* (Table 2). Attack by *P. subgranosus* was initially restricted to wounds or adjacent tissue, but later became more general above and below the wound zone in some infected trees.

TABLE 2—*Chalara australis* infection and *Platypus subgranosus* attack of *Nothofagus cunninghamii* trees (mean dbhob 35 cm), wounded in January 1985, Arve Valley, Tasmania

Treatment	Number of trees	*C. australis* infection category	No. trees in *P. subgranosus* attack class			No. tunnels checked for infection	No. tunnels with *C. australis* infection
			0	<20	>20		
Exposed	24	Infected	2	5(36)*	8(>324)	47	14
		Uninfected	2	6(21)	1(25)	35	0
Covered	12	Infected	1	2(4)	–	4	2
		Uninfected	5	4(27)	–	14	0
			10	17(88)	9(>349)	100	16

* Figures in parentheses indicate total number of *P. subgranosus* tunnels initiated in trees in each infection category/attack class.

Sporulating mycelium of *C. australis* was evident 4–5 weeks after treatment in the moist bases of the exposed wounds of many trees, often before beetle attack. Sampling of some of these infected trees indicated that infection had occurred through the wounds, and that radial streaks of discoloration extended into the stem tissue (Fig. 4). *Ceratocystis moniliformis, C. pilifea,* and *C. piceae* sporulated profusely on the surfaces of hessian-covered wounds, and infection by these species may have contributed to the significant difference ($p < 0.01$) in *Chalara australis* infection frequency between covered (25%) and uncovered wounds (63%). Some *P. subgranosus* attacked the covered wound zones by crawling through or underneath the hessian.

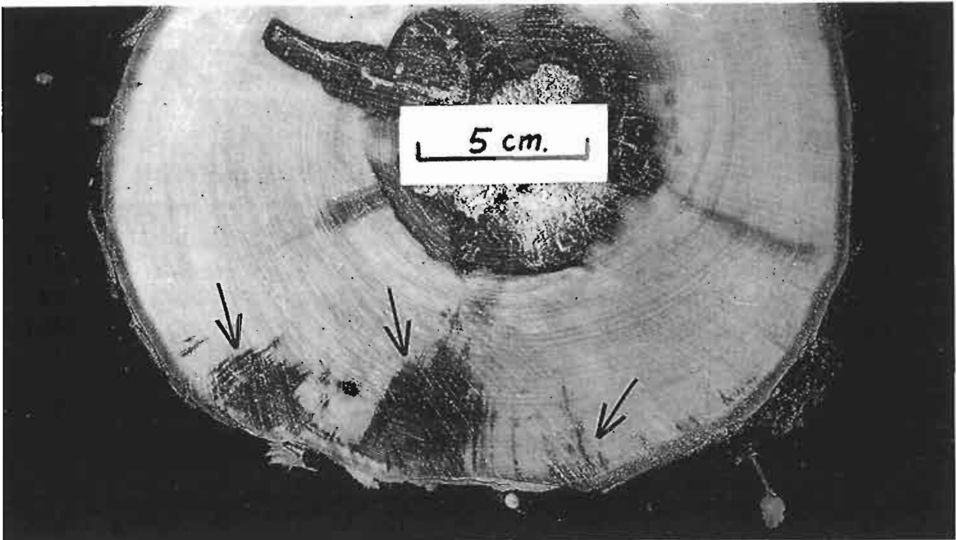

FIG. 4—**Chalara australis** discoloration (arrowed) associated with a stem wound on **N. cunninghamii.**

For Series I billets, as with the wounded trees, there was no relationship between *P. subgranosus* attack and *C. australis* infection; 13 non-attacked billets became infected, while 17 attacked billets remained free of infection (Table 3). Infection of unbagged billets was significantly greater ($p < 0.01$) than bagged billets. *Platypus subgranosus* initiated tunnels on billets within 2–3 weeks of exposure in the forest and there was significantly more attack ($p < 0.01$) at Site E than at Sites A1 or A2 (Table 3). Initial attack concentrated around the ends of the billets, but later occurred more widely over the billet surface. Five bagged billets had some attack (three with one tunnel, one with seven, and one with 23) from beetles penetrating the hessian. Most *C. australis* infections probably originated through billet ends as the extent of discoloration attenuated inwards (Fig. 5).

Although the results from the wounded trees, and from Series I billets, established that *C. australis* infection could occur independently of *P. subgranosus* attack, the high frequency of infection through the billet ends might have obscured a low frequency of dissemination of *C. australis* by *P. subgranosus.* Series II and III billets were used to examine this possibility.

TABLE 3—*Chalara australis* infection and *Platypus subgranosus* attack on *Nothofagus cunninghamii* billets (Series I) exposed from early January 1985 for 9 weeks at three rainforest sites in southern Tasmania

Infection category	Site	Bagged		Unbagged	
		No. billets attacked by *P. subgranosus*	No. billets not attacked by *P. subgranosus*	No. billets attacked by *P. subgranosus*	No. billets not attacked by *P. subgranosus*
Infected					
	Arve 1(A_1)	1(1,1,0)*	2	7(38,14,4)*	6
	Arve 2(A_2)	1(1,1,0)	0	3(27,8,0)	4
	Esperance(E)	2(30,11,1)	1	7(206,68,2)	0
Uninfected					
	Arve 1	1(1,1,0)	0	0	0
	Arve 2	0	3	3(11,7,0)	1
	Esperance	0	2	13(377,133,0)	0
		5(33,14,1)	8	33(659,230,6)	11

* First figure in parentheses indicates the total number of tunnels initiated for all billets, the second the number of tunnels checked for fungal infection, and the third the number from which *C. australis* was isolated.

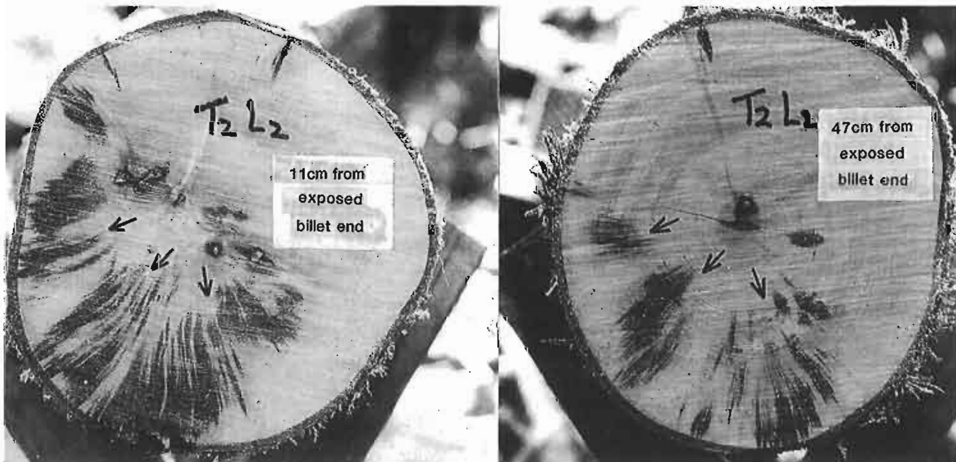

FIG. 5—Attenuation of **C. australis** infection (arrowed) at 47 cm from the end (right) compared with 11 cm from the end (left) of a **N. cunninghamii** billet (Series 1).

Only nine of 38 Series II billets (24% compared with 60% of Series I billets) became infected by *C. australis* (Table 4). There were no significant differences, with respect to *C. australis* infection, between sites or between protected and exposed billets. As there was no significant difference in the mean number of tunnels or in the occurrence of *C. australis* in scorched or water-soaked billets compared with untreated billets, results for all billets were pooled. There was no significant difference in mean attack level on

TABLE 4—*Chalara australis* infection and *Platypus subgranosus* attack on billets of *Nothofagus cunninghamii* (Series II) during the 1985–86 flight season

Site	Incubation treatment	Total billets	Total tunnels	No. billets infected	No. discrete infections	No. tunnels directly entering stained wood
Esperance	Protected	8	589	4(343)*	6	10
	Exposed	8	979	2(306)	3	3
Little Florentine	Protected	11	419	1(40)	1	2
River	Exposed	11	963	2(171)	2	19
		38	2950	9(860)	12	34

* Figures in parentheses are total number of *P. subgranosus* tunnels in all infected billets in each treatment.

infected billets (nine, mean 95.6 tunnels) and uninfected (29, mean 72.1 tunnels). The time from billet exposure to the initiation of *P. subgranosus* tunnels, and the distribution of tunnels in billets, were similar to those in Series I billets.

Seven of the *C. australis*-infected billets had single columns of discoloration, while two had two and three separate infections, respectively. In two billets, infection could have originated via cracks which developed in the ends of billets during the incubation period. Visual inspection of approximately 80% of tunnels and isolation from 10% of these in clear wood in both infected and uninfected billets, did not reveal any further infections. Only 34 tunnels (1.15% of the total number) were found to enter directly wood discoloured by *C. australis* in the nine infected billets.

Chalara australis infection was found in 7/12 drilled billets in Series III, and there was infection among some billets in all treatments (Table 5). Twelve or more separate infections were associated with the drill holes and their length varied from a few centimetres to >70 cm. Some infections could be related to individual drill holes. One untreated billet had a single infection that was associated with a bark wound caused by a poorly occluded branch stub.

TABLE 5—The number of *Nothofagus cunninghamii* billets (Series III) infected by *Chalara australis* and the number of discrete infections (in parentheses) which developed in those billets between the winter and early summer of 1986

Incubation	Unbagged		Bagged		Total
	Untreated	Drilled	Untreated	Drilled	
Protected	1/3 (1)	2/3 (≥5)	0/3	2/3 (4)	5/12(≥10)
Exposed	0/3	2/3 (2)	0/3	1/3 (1)	3/12 (3)
	1/6 (1)	4/6 (≥7)	0/6	3/6 (5)	8/24(≥13)

Isolation of *C. australis* from *P. subgranosus* Frass on Trees and Billets of *N. cunninghamii*

Chalara australis was isolated from 70% of frass samples derived from trees with normal crowns but with current flight season attack by *P. subgranosus* (Table 6, Category A 1). Up to a maximum of about 2 years after tree death, and after a change from adult to larval frass upon hatching of eggs and extension of the tunnels by larvae (Hogan 1948), the frequency of infection of the frass decreased to zero (Table 6, Category A 3–6). The fungus was also readily isolated from frass from three trees inoculated with *C. australis* in December 1985 (Table 6, B). However, *C. australis* was absent from frass from Series II billets exposed to *P. subgranosus* attack over the same period as the *C. australis*-inoculated trees (Table 6, C), or from frass from eight healthy trees that were ringbarked or scorched in December 1985 to induce *P. subgranosus* attack (Table 6, D).

TABLE 6—Isolation of *Chalara australis* from *P. subgranosus* frass on trees and billets of *Nothofagus cunninghamii*, Little Florentine River site, February–March 1986

Category	No. trees or billets	Type of frass		Percentage *C. australis* infection (adult plus larval frass)
		Adult	Larval	
A. Trees naturally attacked by *P. subgranosus*				
(1) Healthy with current attack	6	64/92	0	70
(2) Early symptoms	3	24/44	7/18	50
(3) Dead >70% foliage retained	4	33/40	14/36	62
(4) Dead 20–70% foliage retained	4	13/21	12/49	36
(5) Dead <20% foliage retained	3	0/18	0/27	0
(6) Dead, fine twigs only	9	0/10	0/58	0
		134/225 (60%)	33/188 (18%)	
B. *C. australis*-inoculated trees (healthy with current attack)	3	78/91	0	86
C. Billets exposed to *P. subgranosus* attack	16	0/157	0	0
D. Scorched or ringbarked trees	8	0/55	0	0

Potential for Within-host Contamination by *C. australis* of Pre-emergent *P. subgranosus*

The isolation of *C. australis* from tunnel scrapings and from *P. subgranosus* adults and larvae, as well as the presence of phialophores, were not significantly different within disease categories 1–3 or 4–6 (Table 7). However, there was a significant decrease ($p < 0.001$) for these parameters between the two groups (healthy to recently dead *v.* dead 6 months to ≥ 2 years). Isolations of *C. australis* from wood followed the same trend, but differences between the two disease groups were not significant (Table 7). Position (inner or outer wood) did not influence isolation from tunnel scrapings or presence of phialophores but both parameters were significantly affected by tunnel type (dark > moderate > light) and whether or not tissues were *C. australis*-infected.

TABLE 7–Success of *C. australis* isolation from tunnel wall scrapings, adult and larval *P. subgranosus* (percentage total per category), and wood (mean percentage per category) and phialophore presence in tunnels (percentage total per category) from naturally diseased *N. cunninghamii** in each of six disease categories

Disease category (n=3 per category)	Isolations from tunnel wall scrapings by tunnel type†			Isolation from *P. subgranosus*†			Isolation from wood	Presence of phialophores by tunnel type†		
	Dark	Moderate	Light	Female	Male	Larvae		Dark	Moderate	Light
1.Healthy, current attack	30	52	25	24	27	3	59	27	36	17
2.Symptoms dying	47	25	5	26	39	3	46	35	17	10
3.Dead, >70% foliage retained	43	5	20	29	26	2	46	46	16	40
4.Dead, 20–70% foliage retained	3	0	0	0	4	0	10	6	0	0
5.Dead, <20% foliage retained	0	0	0	0	0	0	11	0	0	0
6.Dead, fine twigs only	0	0	0	0	0	0	0	0	4	0

* Mean dbhob 46.3 cm (range 31–60)

† For tunnel scrapings and presence of phialophores, the mean numbers of tunnels examined in each category per tunnel type were Dark 31, Moderate 21, and Light 21. For female and male adults and for larvae the mean numbers examined per category were 16, 26, and 30, respectively

Isolations of *C. australis* from tunnel scrapings could have been of conidial or mycelial origin but those from beetles are presumed to be due to conidial contamination. *Hormoascus platypodis* and *Raffaelea* sp. were consistently isolated from tunnel wall scrapings in trees of all categories. As well as *C. australis,* most of the species previously recovered from beetles were isolated. In disease categories 4–6, *Leptographium* sp. and xylariaceous and basidiomycetous species were more commonly isolated, paralleling a similar increase in isolation frequency for these fungi from wood. The changes in isolation profile reflected changes in the nature of the tunnel scrapings from light to dark wall type. In light or moderate tunnel types conidiophores of *H. platypodis* and *Raffaelea* sp. were commonly observed, but with darkening of the walls the scrapings consisted of isolated conidiophores, conidia, hyphae, comminuted wood, and amorphous debris. Conidia ($23-(30)-38 \times 18-(25)-29\,\mu m$) of *Sphaerosporium* sp. (Fig. 6) and the unidentified structures (Fig. 3) occurred abundantly in some dark tunnels in trees of disease categories 4–6.

With one exception phialophores were found only in tunnels of infected wood (Fig. 7). While in the initial assessment a distinction was made between viable and non-viable phialides, there were a number of examples of tunnels containing no phialophores (or phialophores judged to be non-viable) from which the fungus was isolated. Hence the data were pooled for statistical analysis. Phialides were not found in any of 23 pupal chambers examined.

Adults of *P. subgranosus* were usually common in the wood of trees of disease categories 1–5, although increased numbers of dead beetles were found in categories

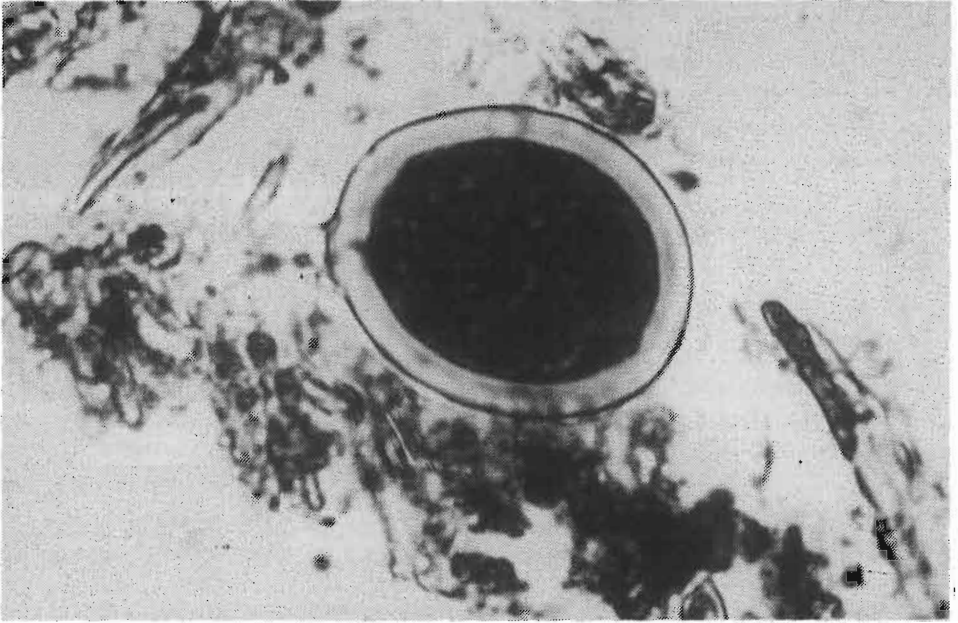

FIG. 6—Conidium of **Sphaerosporium** sp. in **P. subgranosus** tunnel wall scrapings (× 400).

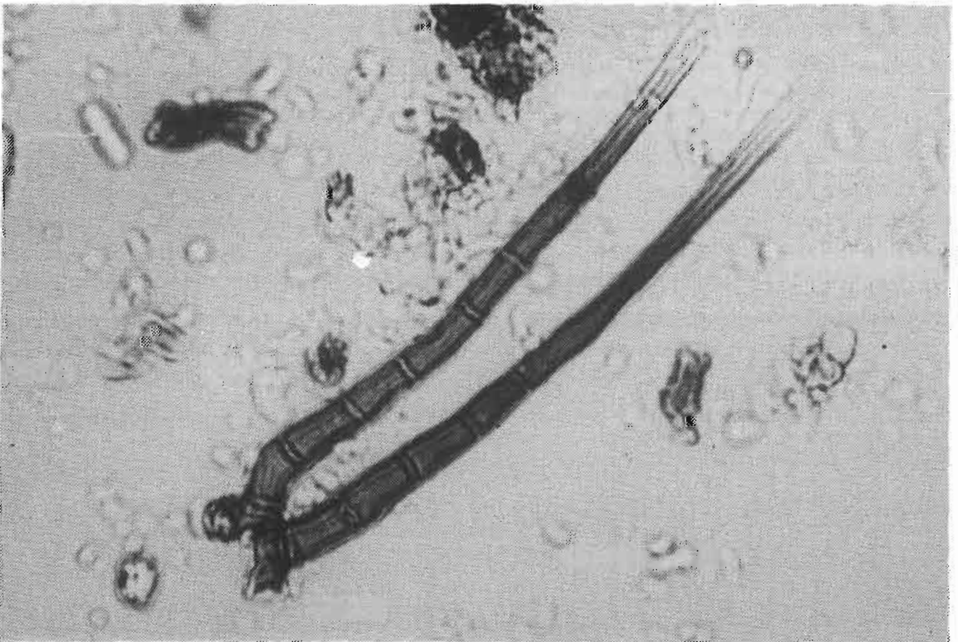

FIG. 7—Phialophores of **C. australis** among scrapings from **P. subgranosus** tunnels (× 200).

4–6 and few living beetles occurred in category 6 trees. *Chalara australis* was isolated from both male and female beetles at approximately the same frequency (20% and 20.5% respectively). Larvae were most abundant in category 3–5 trees, but significantly fewer larvae (3.3%, $p < 0.001$) than adults yielded *C. australis.*

Isolation success from wood varied within discs. Sometimes only restricted areas of tissue yielded the fungus even though *C. australis* discoloration extended around the entire circumference of most discs except those in categories 1–2. *Chalara australis* was often recovered in pure culture from categories 1–3, but an increasing variety of other fungal species were isolated from categories 4–6.

DISCUSSION

It has not been possible to demonstrate for *P. subgranosus* three of the four requirements suggested by Leach (1940) as the minimum for adequate proof of insect transmission of a plant pathogen, although a small percentage of the beetles may be casually contaminated with viable propagules of *C. australis.* Occasional carriage could be expected as beetles breed within wood infected by *C. australis* and may contact external sporulating mycelium of the fungus (Kile & Walker 1987) between emergence and attack of a new host (the two known potential points of contact between the beetle and the fungus). The only time significant numbers of *P. subgranosus* become contaminated with *C. australis* is after the initiation of attack on *C. australis*-infected trees when the fungus sporulates on the walls of tunnels excavated into infected wood. As these contaminated brood parents do not re-emerge from the host (H. J. Elliott pers. comm.), the next generation of adult beetles developing from eggs within the host must become contaminated if *P. subgranosus* is to be a vector. This will be a rare event because the life cycle of *P. subgranosus* is somewhat longer than the saprophytic survival of the fungus in the stem wood of trees from which brood emerges (Fig. 8). Although the length of the *P. subgranosus* life cycle may vary from 10 months

***Platypus subgranosus* life cycle**									
YEAR 1				YEAR 2				YEAR 3	
S	A	W	S	S	A	W	S	S	A

Attack by adults
Eggs
Larval instars
Pupae
Emergence of adults

Host disease category

1–2	3	4	5	6
Chalara australis isolation (%) from frass (and wood) in each category				
70–50 (59–46)	62 (46)	36 (10)	0 (11)	0 (0)

FIG. 8—Generalised life cycle of **P. subgranosus** in relation to host disease category and two measures of **C. australis** viability within the host.

to more than 4 years (Hogan 1948; Elliott pers. comm.) most of the brood emerges in the second summer after attack, by which time *C. australis* survival in the above-ground wood is minimal. The potential for contamination of beetles through contact with patches of external sporulating mycelium also seems likely to be low. Unsuccessful attack in these mycelial patches could result in some free-flying contaminated beetles which might account for the *C. australis* isolations from some free-flying beetles as opposed to the negative results with beetles newly emergent from the host.

The number of *P. subgranosus* available for isolation from in-flight trappings and *in situ* brood trees was much less than that from tented logs, the only method by which large numbers could be obtained. In-flight trappings were unpredictable in relation to both weather conditions and numbers caught and not all beetles trapped in flight necessarily emerged from *N. cunninghamii* trees killed by *C. australis*. The contamination by *Mucor* and *Rhizopus* spp. of beetles from tented billets was probably a consequence of the humid conditions prevailing in the closed tents.

Although no selective medium exists for the isolation of *C. australis*, the evidence indicates that the negative isolation results for the fungus, particularly from the direct plating of emergent beetles and frass from billets, genuinely reflects its absence from the material plated. The high frequency of recovery of *C. australis* from deliberately contaminated *P. subgranosus*, from frass, tunnel scrapings, and from beetles in naturally attacked trees, in the presence of all the suspected ambrosial fungi and a range of other species, indicates the good competitive ability of the fungus in mixed cultures and the reliability of the detection methods used. Inoculation of *N. cunninghamii* seedlings or stem discs with whole beetles, or macerates, aimed to use the fungal host as a selective medium. The results from these isolation attempts were also considered genuine as *C. australis* conidia remained viable in the presence of beetle body contents.

Platypus subgranosus is an exception amongst the 10 *Platypus* spp. so far studied (Roche & Lhoste 1960; Farris & Funk 1965; Francke-Grossmann 1966; Nakashima 1975) in that it apparently lacks mycetangia. As xylomycetophagy is thought to represent a more highly evolved habit (Browne 1961a), lack of specialised organs for the storage and transport of symbiotic fungi suggests that *P. subgranosus* is a relatively primitive ambrosia beetle. The inoculum of the symbiotic associates is probably carried largely on the dorsal cuticle of the body or possibly in mouth parts. Any propagules of *C. australis* which might be carried by *P. subgranosus* are likely therefore to represent external contaminants.

The data from the frass isolation studies demonstrate that the beetles are secondary agents attacking trees already infected by *C. australis*. The differing results of frass isolations from disease category 1 and artificially inoculated trees, compared to ring-barked and scorched trees and Series II billets, which involve activity by subsamples of the beetle population in a single location during the same flight season, can be explained only if category 1 trees were already infected by *C. australis*. That attack follows infection is supported by the observation that in both naturally and artificially infected trees attack frequently commenced on one sector of the stem. Dissection of such trees revealed infection in the initial sector of attack, and that as infection spread around the circumference, attack became more general until tree death. Attack by

P. subgranosus in billets was not related to the occurrence of *C. australis* infection, but was probably more influenced by attractant volatiles emanating from the billets (Elliott *et al.* 1983). The presence of phialophores and phialoconidia of *C. australis* in frass would also explain the development of small patches of sporulating mycelium on the bark around tunnel entrances on some attacked trees. Contaminated frass could also act as windborne inoculum as shown for frass from *Xyleborus* sp. infected with *Ceratocystis fimbriata* (Ell. & Hals.) in cacao (Iton 1959, 1961).

The most probable origin of the infections in the wounded trees and billets is airborne or waterborne inoculum. In wounded trees there was evidence of infection through the moist bases of wounds, and in Series I billets consistent evidence of entry through cracks and poorly sealed ends. The treatment of Series II billets, which attempted to prevent entry of *C. australis* through any point other than the wounds made by beetles, resulted in only 12 infections, 10 of which could have originated via tunnels. The infection of tunnel-like wounds during the non-flight season demonstrated that the infections in Series II billets could not necessarily be ascribed to transmission of inoculum by the beetle. While *C. australis* produces conidia in moist droplets, the infection of exposed billets and those protected from precipitation in the forest raises the possibility of airborne dispersal of inoculum. Transmission by other insects seems unlikely, because none were consistently associated with both healthy and diseased *N. cunninghamii* or with *C. australis* external mycelium. However, no study has been made of insect species visiting fresh wounds in *N. cunninghamii* trees or logs.

In terms of its general life habits and lack of a pathogen vector role, *P. subgranosus* does not differ from the majority of xylomycetophagous platypodids and scolytids (Browne 1961a). Attack by native ambrosia beetles on healthy living trees in natural forest is unusual, the great majority attacking logs and timber. Based on Browne's (1965) classification of the circumstances in which ambrosia beetles infect living trees in tropical forests, only *Dendroplatypus impar* Schedl which infests *Shorea* spp. in Malaysia (Browne 1961a), *Trachyostus ghanaensis* Schedl which infests *Triplochiton scleroxylon* K. Schum in West Africa (Roberts 1960, 1968), *Austroplatypus incompertus* (Schedl) Browne which infests at least 13 *Eucalyptus* spp. in Victoria and New South Wales (Schedl 1968; Browne 1971; Harris *et al.* 1976), *Doliopygus dubius* Samps. which infests *Terminalia superba* Engl. *et.* Diels (Browne 1961b), and possibly *Platypus* spp. which infest *Nothofagus* spp. in New Zealand (Milligan 1974; Faulds 1977) are known to cause habitual and persistent attack on apparently healthy trees, or sporadic attack on healthy trees. However, examples are frequent of infestation in trees and shrubs of low vigour, through wounds, or of unhealthy and dying trees. These include many reports of attack by indigenous ambrosia beetles on exotic tree species or vice versa (Kalshoven 1960; Browne 1961a). No ambrosia beetles have been shown conclusively to transmit pathogenic fungi regularly although it has been suggested, or inferred, that some have (Moore 1959, 1962; Iton 1960; Ngoan *et al.* 1976; Faulds 1977; Weber & McPherson 1984a, b). For oak wilt caused by *Ceratocystis fagacearum* Bretz, a number of ambrosia beetles were excluded as vectors even though some of them carried viable spores on emergence from diseased trees (Stambaugh *et al.* 1955; Wertz *et al.* 1971). This lack of a pathogen vector role for ambrosia beetles contrasts with the phloeophagous scolytids (bark beetles) for which such a function is relatively common

(Browne 1961a). Our evidence supports the hypothesis that *P. subgranosus* is a secondary factor in myrtle wilt, with the beetle attacking trees already infected by *Chalara australis*. How the trees become infected initially, and why beetles selectively attack such trees, is the subject of further studies. These include evaluation of an alternative hypothesis that *P. subgranosus* may attack stressed *N cunninghamii* trees which then become infected by *C. australis* via the *P. subgranosus* tunnels. On the basis of our study, a role for *Platypus* sp., in dispersing *Sporothrix* spp. the cause of death of *Nothofagus* spp. in New Zealand (Faulds 1977), could be considered doubtful.

A small percentage of *P. subgranosus* may carry and transmit *C. australis* although the latter is probably an uncommon event. Given the apparent propensity for airborne/waterborne infection of wounds by *C. australis,* and the occurence of below-ground spread (Elliott *et al.* 1987; Kile unpubl. data) it is likely that *P. subgranosus* contributes little to the spread of myrtle wilt.

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