

THE MECHANISM OF PATHOGENICITY OF *SIREX NOCTILIO* ON *PINUS RADIATA*

I. EFFECTS OF THE SYMBIOTIC FUNGUS *AMYLOSTEREUM* SP. (THELOPHORACEAE)

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Summary

Effects on *P. radiata* of the fungus, *Amylostereum* sp., which is injected into the sapwood of the stem by *S. noctilio*, are described. The fungus causes the sapwood to dry out in advance of the mycelium, and tests were made to find whether it kills the tree by restricting sap flow to the crown, or by producing toxins. No systemic toxic effects could be attributed to the fungus, which appeared to act by restricting sap supply, but rapidly developing systemic effects were observed after experimental *S. noctilio* attack, implying that a secretion of the insect was involved in pathogenicity.

I. INTRODUCTION

The wood wasp, *Sirex noctilio*, occurs in symbiosis with a fungus recently classified as an undetermined species of *Amylostereum* (Talbot 1964). The insect attacks its host tree which, in Australia and New Zealand, is chiefly *Pinus radiata* D. Don, by drilling into the stem with its ovipositor. Eggs, fungal arthrospores, and certain secretions from the female insect are injected into the sapwood to a depth of about 1 cm.

Amylostereum sp. appears to be a weak parasite and many trees resist attack, but under some conditions damage can be considerable and Rawlings (1954) records that, in New Zealand, *S. noctilio* attack was responsible for the death of 30% of the trees over approximately 600,000 acres of *P. radiata* forest.

The often lethal nature of *S. noctilio* attack has for a long time been attributed to the effects of the fungal symbiont, but its mode of action has remained obscure. It has been suggested that growth of the fungus cuts off the sap supply to the tree's crown (Rawlings 1948), and certain blue-stain fungi carried by bark beetles are thought to kill trees in this way (Caird 1935; Mathre 1964). Attack by *S. noctilio* or inoculation of trees with *Amylostereum* sp. certainly dry the sapwood locally to an extent which would render it non-conducting (Coutts 1965; Coutts and Dolezal 1965). Attacked trees are usually inoculated by the insect all round the stem, and if dry zones in the sapwood coalesced, the sap supply to the crown would be cut off. However, physiological changes occur in the stems and leaves of trees very soon after attack, even in resistant trees, in which the fungus grows only about 6 mm in the first 2 weeks,

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there being no further spread after 3–6 weeks (Coutts and Dolezal 1966). Although restriction of sap flow could well account for the final death of the tree, it could scarcely explain the early symptoms of the disease, and a hypothesis was put forward that physiological changes occurring during the first 2 weeks after attack were induced by some substance or substances, translocated from the site of attack, and having a powerful systemic effect on the entire tree (Coutts 1968). To investigate this hypothesis typical patterns of tree reaction were observed, and tests were made to find whether these could be caused by impeded sap flow, or by injecting extracts of fungus-infected material into trees.

II. MATERIALS AND METHODS

Only techniques repeatedly used are described in this section. Experimental details concerned with particular investigations are given before the results of each experiment.

(1) *Experimental Trees*.—Trees used were in two areas of *P. radiata* regrowth, 5–7 and 14–16 years old, at Pittwater Plantation in southern Tasmania.

(2) *S. noctilio* *Attack*.—Mated insects with glued or clipped wings were used on pruned parts of the trees' stems, the attack sometimes being restricted to defined areas.

(3) *Inoculation*.—*Amylostereum* sp., originally isolated from the intersegmental sacs at the base of the ovipositor of the female insect, was grown on lumps of autoclaved *P. radiata* sapwood, half immersed in water. After 6–8 weeks incubation, the flasks were shaken to separate the mycelium, which was used in suspension in distilled water. Boreholes 3 mm in diameter, drilled into the sapwood, were filled with this suspension, and sealed with cellulose tape.

(4) *Preparation of Log Extracts*.—Extracts were obtained from *P. radiata* logs 4 ft long, which had been incubated for various times after attack by *S. noctilio* or inoculation with *Amylostereum* sp. Distilled water from a reservoir attached to one end of each log was drawn through the log by applying a vacuum to the other end.

(5) *Injections of Solutions into Trees*.—Small quantities of solutions were injected into boreholes in the stem with a syringe. Large quantities were fed into boreholes through a pipe connected by tubing to a reservoir above.

(6) *Moisture Content Determinations*.—Moisture content (MC) of bark, wood, and leaf was determined as a percentage of dry weight (MC/D), and as a percentage of water present at saturation (MC/S). Bark MC was measured by a method similar to that used on poplars by Bier (1961). Wood MC was measured either on cores cut from standing trees with a core-cutting bit, or on samples split from disks sawn from felled trees. After weighing, wood samples were saturated with water under vacuum or by boiling, as the latter gave the same result much more quickly. For leaf MC, the water-deficit method of Stocker (1929) as modified for pine needles by Rutter and Sands (1958) was used, one sample of 10 2-yr-old fascicles and one sample of 10 1-yr-old fascicles being taken from each tree at sunrise.

(7) *Starch in Leaves*.—A visual estimation was made of starch in the leaves, similar to that described by Rutter and Sands (1958), but instead of examining sections of the needles, the latter were cut across at a point midway along their length, and after staining in iodine–potassium iodide, the cut ends were examined with a stereomicroscope. This allowed the examination of far more samples than was possible when the slower method involving sections was used. To reduce subjective error, assessments were made by an observer who did not know from which trees the numbered samples had been taken. Seven grades were recognized, from grade 0 at which no starch could be detected, through grade 1 (trace), up to grade 6 (mesophyll packed with starch grains). Six 1-yr-old and six 2-yr-old needles from each tree were examined at each assessment.

(8) *Leaf Dry Weight and Density*.—Differences in leaf dry weight of sequential samples were determined by cutting an exact 5-cm length from a bunch of fascicles with a special tool, and weighing after drying at 95°C for 10 hr. A sample consisted of 10 fascicles, two being picked from each of the same five branches on each tree, at each time of measurement. For density determinations, leaf volume was measured by displacement of water containing detergent in a burette.

III. RESULTS

(a) Changes in the Appearance of the Foliage after Experimental S. noctilio Attack

After *S. noctilio* had been induced to attack the stems of healthy trees distinct symptoms were observed in the foliage. A moderate to heavy attack was nearly always followed 2-4 weeks later by yellowing of the old needles attached to the stem and lower parts of branches, the basal part of the needles being the first to show the effect. When trees resisted attack, the rest of the foliage usually remained green, or failed to change beyond an olive green colour. When trees were unable to resist the attack, either the yellowing and premature abscission extended to include the needles near buds, or the preliminary yellowing of 2- to 3-yr-old needles was followed by a period of a month or more during which no visible change took place, the remainder of the crown dying later, and often being marked by collapse of the younger needles while they were still a green or grey-green colour.

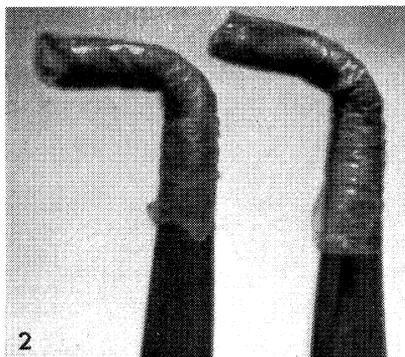
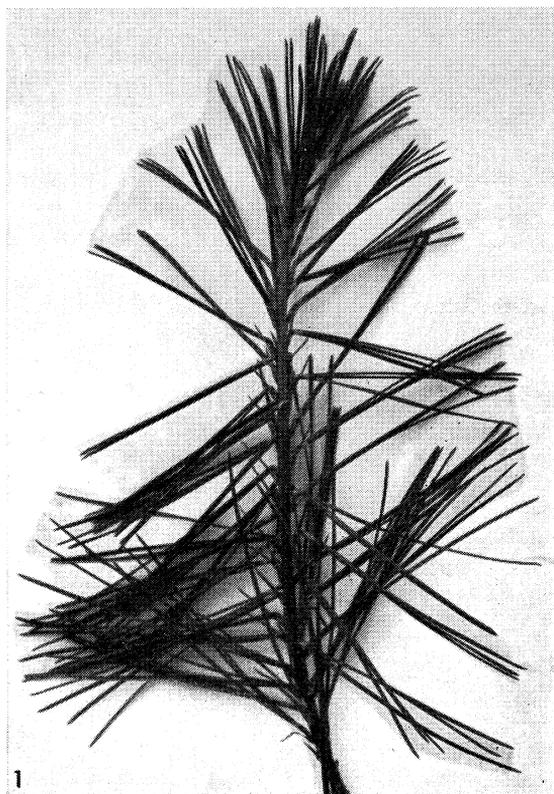


Fig. 1.—Shoot from near the top of an attacked tree, showing wilting of some of the needles near the tip.

Fig. 2.—Typical wilted needles which have bent about half-way along the part covered by the fascicle sheath.

The progressive yellowing of needles by age classes, from the oldest to the youngest, was sometimes accompanied by a sudden wilting of the young needles close to the tips of branches in the upper part of the crown (Figs. 1 and 2). Trees in this condition rarely recovered. Needles in which the meristem was still active were particularly prone to wilt, and *S. noctilio* attack in late autumn and during periods of drought was more rarely followed by wilting.

(b) *Exploratory Work to Investigate Restricted Sap Flow*

If action of the fungus was cutting off the sap supply to the upper part of the tree, bark MC should decrease above the attacked zone, as it is sensitive even to hourly changes in moisture stress. A tree 30 ft tall was given heavy *S. noctilio* attack on the lower 7 ft of its stem. Four weeks after the attack, when half of the crown was yellow, MC of the bark of the attacked tree and of an unattacked control tree was measured (Table 1). No decrease in bark MC/S occurred above the attacked zone. The sapwood had been dried locally at a height of 7 ft, but the wood MC/S was unaffected above the attacked zone.

TABLE 1
MOISTURE CONTENT AS PERCENTAGE OF WATER PRESENT
AT SATURATION (MC/S) OF BARK AND WOOD 1 MONTH AFTER
S. NOCTILIO ATTACK

Height of Sample (ft)	MC/S of Attacked Tree*		MC/S of Control Tree	
	Bark	Wood†	Bark	Wood‡
0·5	60	82	58	85
5	68	74	56	83
7‡	76	25	55	84
10	54	81	55	81
15	51	80	51	81
20	57	81	57	81

* Attacked to a height of 7 feet.

† Outer 1 cm of sapwood.

‡ Height where maximum attack occurred.

These results suggested that 4 weeks after *S. noctilio* attack, when half the foliage was dead, the fungus had not effectively reduced the water supply to the crown of the tree. However, trees sometimes survive *S. noctilio* attack heavy enough to kill one side of the stem, although this results in the death of some of the foliage. Moisture relations of localized attack were therefore investigated.

S. noctilio attack was confined to a rectangular area 2 ft long and 6 in. wide, at a height of 3–5 ft, on two trees 30 ft tall and 4 in. in diameter at breast height. The foliage of both trees began to turn yellow after 3 weeks. One tree was then attacked by wild *S. noctilio* right up its stem. The other tree received no wild attack, and after 1 month, when three-quarters of the crown was yellow, the MC of bark and wood was measured. There was a marked reduction in wood MC/S in the attacked zone only (Table 2), and fungal drying extended only 1·5 cm radially.

As a further control, two trees were each sawn half way through at heights of 3 and 5·5 ft, and the wood between the cuts was chiselled out. This drastic treatment had no visible effect on the foliage, so the amount of obstruction to the sap stream in the attacked zone of the locally attacked tree could not account for the death of the crown.

(c) *Changes in Leaf Properties and the Effects of Shading*

Trees most commonly killed by *S. noctilio* are those in the lower crown classes, and as shading is a major cause of suppression, shaded trees were included in this experiment on changes in leaf properties.

Thirty-six 5-6-yr-old trees with a mean height of 10 ft and mean diameter of 1.5 in. were allotted to the treatments shown below:

Treatment	No. of Trees	Treatment	No. of Trees
Unshaded		Shaded	
Control (no <i>S. noctilio</i> attack)	6	Control (no <i>S. noctilio</i> attack)	6
Heavy attack	8	Light attack	8
Light attack	8		

Three days before the attack, the crowns of trees to be shaded were covered by hessian, which intercepts 75-85% of light.

S. noctilio were restricted to the lowest 2 ft of the stems, six insects being used for heavy attack and three for light attack. (Most insects were removed after 1 day, although some had to be left for an additional day to give what was judged to be an even amount of attack within the heavy- or light-attack treatments.)

TABLE 2
WOOD AND BARK MOISTURE CONTENT AS PERCENTAGE OF WATER PRESENT AT SATURATION (MC/S)
1 MONTH AFTER LOCALIZED *S. NOCTILIO* ATTACK

Treatment	Height of Sample (ft)	Position of Sample	Bark MC/S	Wood MC/S
Local attack	5	Attacked area	64	25
	5	Opposite side	60	81
	18	Above attacked area	59	81
Local attack plus wild attack*	5	Attacked area	50	30
	5	Opposite side	56	75
	18	Above attacked area	50	75
Control	5		61	78
	18		57	80
Control	5		54	82
	18		57	79

* Heaviest attack between height of 3 and 5 ft in "attacked area"; lightly attacked elsewhere.

Many 2-3-yr-old needles on the attacked trees died during the second week following the attack, but by the third week no further yellowing occurred, except on five trees which died. Four of these were shaded, the fifth being a lightly attacked unshaded tree. Some 6 months later, a further shaded attacked tree died, making a total of 5 out of 8 lightly attacked shaded trees which died, compared with 1 out of 16 unshaded attacked trees, 8 of which had heavy attack.

Leaf MC was measured at intervals (Fig. 3). The yellowing of old needles on trees which resisted the attack was not accompanied by any decrease in leaf MC/S, either in the young needles, which remained green, or in the yellowing needles themselves, indicating that the disappearance of chlorophyll which preceded the death

of the old needles was not due to restriction of sap flow in the stem. However, 3 weeks after the attack, the first five trees which died showed a sudden decrease in leaf MC/S, and the leaf MC of these trees continued to decrease as they died during the weeks which followed.

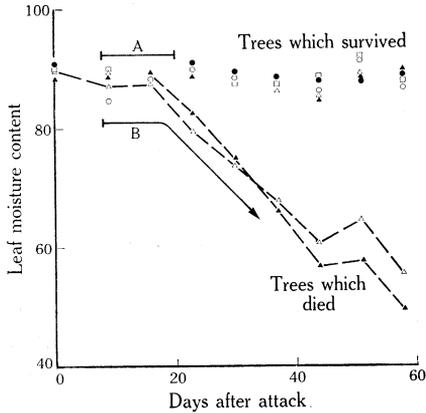


Fig. 3.—Leaf moisture content (as a percentage of the water present at saturation) of trees attacked by *S. noctilio*. A, period of yellowing of 2- and 3-yr-old needles on trees resisting attack. B, Progressive yellowing of trees which died.

Symbol and Meaning	No. Trees Survived	No. Trees Died
○ Control	6	0
● Shaded control	6	0
△ Light attack	7	1
▲ Shaded light attack	4	4
□ Heavy attack	8	0

Although trees which resisted the attack showed no decrease in leaf MC/S, the eight heavily attacked trees showed a pronounced decrease in leaf MC/D 9 days after the attack. When leaf density was measured, it was found that leaves of these trees had increased in density by an average amount of 11%, as the following tabulation shows:

	Heavy Attack	Control
No. of trees	8	6
Mean leaf density:		
0 days after attack	0.352	0.345
9 days after attack	0.391**	0.342

** Increase significant at the 1% level.

This increase in leaf density fully accounted for the decreased MC/D of these trees.

It was supposed that the increased leaf density of the attacked trees might be due to the accumulation of photosynthate in the leaves. Seventeen days after attack, refractometer readings of sap expressed from leaves of five trees, taken at random from each treatment, showed higher values in attacked trees than in controls, as the following tabulation shows:

Treatment	Refractometer Reading*
Unshaded heavy attack	24.8
Shaded light attack	24.8
Unshaded light attack	23.2
Unshaded control	21.4
Shaded control	16.0

* Values are shown as percentage sucrose, and are means for five trees per treatment. Bracketed values differ significantly at the 1% level.

After 3 weeks, leaf starch of heavily attacked and control trees was measured. The attacked trees had far more starch (grades 4-7) than the controls (grades 1-2).

(d) Accumulation of Substances in the Leaf

The starch accumulation in the leaf was investigated on 12 trees similar to those in the previous experiment. Six were given heavy *S. noctilio* attack on the lower part of their stems, and six were kept as unattacked controls.

Starch in the crowns of all the attacked trees increased rapidly to a level at which nearly all leaves were densely packed with starch grains 9–14 days after the attack (Fig. 4). Leaves of control trees did not have much starch at any time, although a minor peak, which followed a slight decrease, was shown during the second week after the trees were pruned. Later work has suggested that this may have been a response to the injury of pruning rather than to environmental conditions. Starch in the leaves of the six attacked trees decreased as the trees died during the weeks which followed.

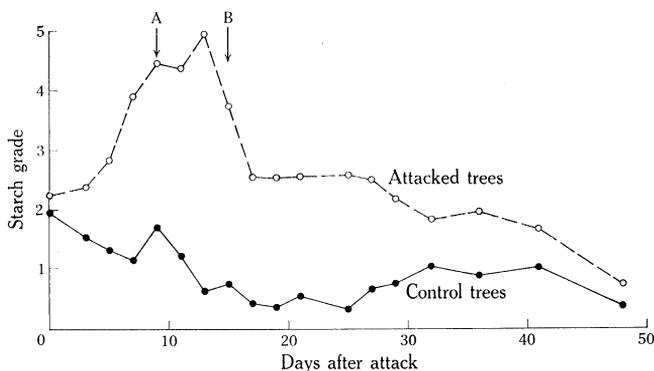


Fig. 4.—Mean starch levels [measured subjectively as described in Section II (7)] of leaves of six trees which died after *S. noctilio* attack, and of six control trees. A, Time of attack by wild *S. noctilio*. B, Time of rapid decrease in leaf moisture content.

To quantify the accumulation of substance in the leaves, leaf density was measured, and it was found that *S. noctilio* attack caused a mean increase of 13% in 2 weeks, as shown in the following tabulation, where the values are means for six trees:

	Attacked Trees	Control Trees
Leaf density 0 days after attack	0.354	0.352
Leaf density 14 days after attack	0.400**	0.344

** Increase significant at the 1% level.

That the accumulation of starch in the leaf is not restricted to trees which die of attack was demonstrated in a later experiment, in which *S. noctilio* attack was induced on 18 trees, 11 of which survived whilst 7 died. Starch accumulated in the leaves of trees which resisted the attack at much the same rate as in those which died, and later it returned to the level of starch in leaves of control trees.

To find whether the accumulation of carbohydrate in the leaf was due to movement of substances into the leaf from the stem, a branch on each of five attacked trees was shaded with black plastic sheeting, and a similar branch was covered with clear plastic. Starch accumulated only in leaves on the latter and on uncovered branches, so the increase in starch was presumably due to the accumulation of current photosynthate.

From the foregoing, it is evident that very soon after *S. noctilio* attack on the stem, photosynthate accumulates in the leaf and this is not accompanied, during the

first 2 weeks, by any decrease in leaf MC/S. When considered in conjunction with the fact that the fungus usually grows no more than 1 cm in resistant trees, this suggested the activity of some substance translocated from the site of attack. To test this hypothesis, extracts of *S. noctilio*-attacked and of inoculated logs were fed into the sap stream of healthy trees, and measurements were made of changes in the leaf.

(e) *Effects of Extracts from Logs which had been Attacked by S. noctilio, or Inoculated with Amylostereum sp.*

Trees used had been topped to leave a single whorl of branches, in order to increase the concentrations of extracts reaching the foliage.

In the first experiment of this series, two logs were cut from one tree; one was given heavy *S. noctilio* attack, and the other kept as an unattacked control. Two months later extracts from the logs were fed into two healthy trees at the rate of 50 ml of extract per tree per day for 5 days, one tree (A₁) having extract from the attacked log and one (C₁) from the control log. *S. noctilio* were induced to attack a third tree (A₂).

The amount of starch in leaves of trees A₁ and A₂ increased at a similar rapid rate during the 2 weeks which followed (Table 3), whereas tree C₁ remained normal.

TABLE 3
EFFECT OF INJECTING WATER EXTRACTS OF *S. NOCTILIO*-ATTACKED LOGS ON AMOUNT OF LEAF STARCH AND LEAF DRY WEIGHT

Tree No.	Treatment	Leaf Starch Grade at:			
		0 Days*	7 Days*	14 Days*	21 Days*
A ₁	Attacked log extract	1.5	3.0	6.0	6.0
A ₂	Tree attacked	1.0	2.5	5.0	5.5
C ₁	Control log extract	1.0	1.5	1.0	1.0

Tree No.	Treatment	Leaf Dry Weight† at:		Percentage Change
		0 Days*	10 Days*	
A ₃	Attacked log extract	0.650	0.715	+10
A ₄	Attacked log extract	0.602	0.692	+15
C ₂	Control log extract	0.588	0.571	-3
C ₃	Control log extract	0.610	0.579	-5
C ₄	No treatment	0.689	0.669	-3
C ₅	No treatment	0.706	0.698	-1

* Days after attack.

† Expressed as grams per 50 cm of fascicle length.

Extracts from two *S. noctilio*-attacked logs were then injected into two trees (A₃ and A₄) and extracts from two control logs into two other trees (C₂ and C₃). Leaf dry weight of these, and of two untreated trees C₄ and C₅, was measured. Extracts of attacked logs caused a substantial increase in leaf dry weight after 2 weeks (Table 3).

Visible changes in the foliage of trees injected with extracts from *S. noctilio*-attacked logs were similar to those in trees attacked by *S. noctilio*. Control extracts had no visible effects.

Effects of extracts from *S. noctilio*-attacked logs were at this stage thought to be due to substances produced by *Amylostereum* sp., or by the tree or log in response to activity of the fungus. A related fungus, *Stereum purpureum*, which is the causal agent of the silver leaf disease of fruit trees, is known to cause starch accumulation in the leaf (Tetley 1932), and at least some of the changes in the leaf have been thought to be brought about by a toxin translocated from the fungus growing in the stem (Naef-Roth, Kern, and Toth 1963). To isolate the effects of *Amylostereum* sp. from any changes brought about by secretions of the insect, extracts of logs which had been inoculated with suspensions of *Amylostereum* sp. mycelium were used. Five inoculated logs were extracted after 2 months' incubation, and the extracts fed into the sap stream of five trees. Extracts of control logs (drilled as aseptically as possible, but not inoculated) were fed into five control trees. The log extracts were used at the rate of 50 ml per day for 8 days. Eighteen further trees were inoculated with *Amylostereum* sp.

None of the treatments caused any change in leaf dry weight relative to the controls during the 3-week period over which measurements were made. However, three of the trees with extracts from inoculated logs and six inoculated trees died after about 3 months. The needles of trees injected with extracts of inoculated logs changed from green to brown from their tips downwards in a manner quite uncharacteristic of *S. noctilio* attack, and foliage of inoculated trees which died changed to a faded green colour, and shrivelled. Trees injected with control extracts showed, with one exception, no visible effect. The exception was one of the trees injected with extracts from a control log, and it was found that the log had become heavily contaminated by wood-rotting fungi.

IV. DISCUSSION

Although experimental *S. noctilio* attack causes the sapwood to dry out some distance ahead of the wood infected by *Amylostereum* sp., a limited amount of attack had no effect on the MC of bark or wood a few feet above the attacked zone. But it still gave rise to symptoms of attack in the foliage.

Attack caused a decrease in leaf MC/S only in trees which subsequently died. This would be expected if the fungus were killing the tree by restricting sap supply, but it was preceded by changes in the leaf for which restricted sap flow provides no adequate explanation. Starch accumulation and chlorophyll breakdown occurred in foliage of resistant trees; and in trees which died, these changes occurred earlier than any decrease in leaf MC/S.

Fungal toxins were originally suspected of causing these effects, but starch accumulation and the concomitant increase in leaf dry weight which followed both *S. noctilio* attack and injections of extracts of *S. noctilio*-attacked logs, could not be induced by inoculations of *Amylostereum* sp., or by extracts from logs inoculated with this fungus, which had only a non-specific necrotic effect. This pointed to secretions of the insect as being responsible for early changes registered in the leaf. Investigations of this aspect will be reported in the second paper of this series.

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