

# PHYSIOLOGICAL EFFECTS OF MUCUS FROM THE WOOD WASP, *SIREX NOCTILIO* F., ON THE FOLIAGE OF *PINUS RADIATA* D. DON.

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The effects of mucus from *S. noctilio* on the physiology and biochemistry of needles of *P. radiata* are described. Some dramatic changes in respiratory rate and mode are noted, as well as effects on the activity of peroxidase and amylase enzymes. These changes are correlated with the appearance of the characteristic symptoms associated with altered water balance within the needle tissues. The similarity between changes caused by the mucus and natural and induced leaf senescence is discussed.

## I. INTRODUCTION

When the wood wasp, *Sirex noctilio* F. (Hymenoptera: Siricidae) attacks radiata pine trees, *Pinus radiata* D. Don., it elicits a complex syndrome of responses within the tree. These are described in detail by Coutts (1969a, 1969b). A series of rapid physiological changes becomes manifest within 10–14 days of attack; these have been shown by Coutts (1969b) to be caused solely by the wasp's mucus secretion. They can be induced by artificial injection of the mucus into the tree, i.e. without the direct participation of the wasp and without the simultaneous introduction of the fungal symbiont *Amylostereum areolatum* (Fr.) Boidin (Gaut 1969) into the oviposition hole.

Some of the systemic responses, e.g. increases in dry weight of foliage and general retardation of growth, may ultimately be reversed in healthy trees, but those more susceptible to *S. noctilio* attack may lose chlorophyll, followed by premature and complete abscission of the foliage. The mucus alone does not cause tree death but, rather, death follows from the combined effect of "mucus conditioning" and eventual invasion of the sapwood by the symbiotic fungus.

In these experiments we have attempted to characterize and quantitate some of the immediate physiological and biochemical changes in *P. radiata* needles which result from mucus treatment.

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## II. METHODS

### (a) Experimental

The effects of artificial application of mucus were investigated by (i) injection of mucus into whole trees; and (ii) uptake of mucus solutions by excised twigs.

#### (i) Whole Tree Experiments

These were conducted on vigorous (5–6 yr old) *P. radiata* ramets chosen from cloned material grown at the Forest Research Institute Field Station at Hobart. Four trees were each injected with 10 ml of mucus solution (5% in deionized water) using the method of Coutts (1969b). Control trees were injected with 10 ml of deionized water. The effects of this treatment were investigated in 1-yr-old needles taken from branches of the third and fourth whorl behind the shoot apex. Random samples of needles were taken at periodic intervals extending from 3 weeks before to 7 weeks after the mucus injection. Because the appearance of effects was rather irregular along the length of the needles, with a broad tendency to a progression from apex to base, our investigations were concentrated on a standard 1-cm segment from the base of the fascicle.

#### (ii) Experiments on Excised Twigs

Branch tips (about 50 cm in length) were selected from trees which had previously been shown to have high susceptibility to the mucus. Each of six twigs in every trial was allowed to take up 10 ml of 1% mucus solution through the ends of the cut stem. They were then stood in deionized water in glass containers for the duration of the experiment, the water being replaced regularly. Control twigs were stood in deionized water throughout. In some experiments mucus was autoclaved at 15 lb/in<sup>2</sup> for 15 min before use.

### (b) Analysis

At harvest, duplicate lots of 1-cm segments from 50 needles removed from trees (or twigs) of like treatments, were weighed and their respiration rate in darkness was determined in a Warburg apparatus at 25°C. The same needle segments were then used to prepare extracts for further examinations.

#### (i) Cold-water Extracts

The 50 needle segments were ground with sand in 10 ml deionized water, and centrifuged at 20,000 g for 10 min. The supernatant, after concentration to 0.5 ml (by dialysis, using polyethylene glycol, mol. wt. 4000), was used in the assay of various enzyme activities and for electrophoretic investigation. In excised twig experiments the extracts used were not concentrated.

The cold-water extracts were used in the following assays:

- (1) Soluble protein content was measured by the method of Lowry *et al.* (1951) and by the biuret reaction (Layne 1957).
- (2) Peroxidase was assayed spectrophotometrically by the procedure of Machly and Chance (1955), but with *o*-dianisidine as substrate ( $1.25 \times 10^{-3}$  M in 0.1 M acetate buffer, pH 4.5).
- (3)  $\beta$ -amylase was assayed by the method of Bernfield (1955).
- (4) Electrophoresis on acrylamide gels was carried out according to the procedures of Mills and Crowden (1968).
- (5) The residue from the cold-water extraction was assayed for chlorophyll by the method of Arnon (1949), but using 80% ethanol.

#### (ii) Hot-water Extracts

A second batch of 50 needle segments was homogenized with 10 ml of deionized water and boiled for 2 hr. The slurry was cooled and centrifuged at 1000 g for 10 min and the super-

natant stirred for 1 hr with 1 g each of Zeokarb-225, acetate, and Dowex-1, H<sup>+</sup>. The hot-water extracts were used for the following assays:

- (1) Soluble sugar content was determined using the anthrone method of Trevelyan and Harrison (1952).
- (2) The complement of sugars in the supernatant was examined by thin-layer chromatography on cellulose (Whatman CC 41) using the solvent systems n-butanol-acetic acid-water (4 : 1 : 5 v/v) (upper layer) and n-butanol-benzene-pyridine-water (5 : 1 : 3 : 3 v/v). Authentic sugar samples were used as references. Visualization was achieved by using aniline hydrogen phthalate and anisaldehyde-sulphuric acid sprays (Hough 1954).

### (iii) Anatomical Studies

Transverse sections 22  $\mu$ m thick were cut on a freezing microtome from the same needles used for cold-water extracts. These sections were removed from the region 0.5 cm below the standard sample segments. Examination was made of tissues, particularly the phloem, after the sections had been stained in tannic acid-ferric chloride-lacmoid stain (Cheadle *et al.* 1953). A count of starch grains was made on the stellar and endodermal tissues of the needle sections after staining in iodine-potassium iodide. For this purpose an average count was obtained in four separate fields per section at 600 magnifications. Twenty needles per sample, taken at random, were examined for both mucus-treated and control trees.

### (iv) Statistical Analysis

Comparisons made between sets of data obtained over specified time intervals, before and after mucus treatment, have been analysed by the *t*-test. Values of *P* are shown in the legends to the appropriate figures.

## III. RESULTS

Several preliminary investigations of the mucus effect have been conducted since 1967. These trials showed that trees varied appreciably in their response to mucus treatment, just as trees in the field display a varying susceptibility to attack and injury by *S. noctilio*. The results reported in this paper for whole trees derive essentially from an experiment commenced in the early spring of 1969, in which trees of high susceptibility were used. The twig experiments were conducted during autumn 1971. During the first week after mucus treatment some differences were apparent between the results obtained for twigs and those for intact trees. It is thought that these differences are due to physical damage caused by the removal of twigs from the trees, since both control and experimental twigs showed discrepancies of the same order. Within a week or so after twig excision, the levels of most measured parameters in the control twigs tended towards those of control trees.

The treated trees and twigs developed yellow, dry needles which abscised easily (cf. Coutts 1969*b*). In whole trees, progressive defoliation occurred above the injected region, branches below this region being apparently unaffected. The old needles on affected branches abscised earlier than young ones, and apical buds often wilted within about 3 months of treatment. During the period of the experiments, control trees and twigs remained green and without defoliation.

Progressive changes in the measured characteristics are illustrated in the figures. Figure 1 shows changes in the weights of needle segments following mucus treatment. It is difficult to account for the persistent decline in the weight of control needle

segments (tree experiments). We have assumed that it is related to the continued pruning of the trees during the course of the experiment, or that it reflects some seasonal change. Despite this uncertainty, the effect of mucus treatment on needle weight is clear. A corresponding decline in weight of control needle segments was not apparent in the twig experiments. However, these experiments were conducted under more uniform environmental conditions in a glasshouse. In whole-tree experiments the dry weight of needle segments increased [Fig. 1(a)]. However, with the excised twigs treatment with mucus appeared to have no effect on needle dry weight [Fig. 1(b)]. Instead, twig needles tended to lose water more rapidly and to a greater extent than did needles on intact trees [Figs. 1(c) and 1(d)]. Thus, although the dry weight/wet weight ratio of needle segments was increased by mucus in both tree and twig experiments [Figs. 1(e) and 1(f)], the reasons for the increases are different.

Changes in the weight of needle segments were associated with anatomical disturbances, particularly in the phloem, where severe physical distortion and collapse of cells became evident within a few days of mucus treatment. Figures 5–9 show progressive stages in the desiccation and collapse of phloem and other non-lignified elements of the needles, e.g. ray parenchyma, mesophyll, endodermis, and secretory cells lining the resin canals.

Coutts (1969*b*) reported that starch accumulated in the endodermal and mesophyll tissues of the needles during the first 10 days after mucus treatment. Our results have not confirmed this observation, and show instead that the starch content of needles is reduced by mucus (Fig. 2). Thus, in experiments with whole trees, the starch content of leaf cells was shown to fall—slowly during the first week, but rapidly thereafter. In the experiments with twigs, the act of excision alone caused a substantial fall in the starch content of both treated and control needles alike. However, after some 3 weeks control needles began to increase their starch content, but the level in needles of mucus-treated twigs remained low.

That mucus affects the chlorophyll content of needles is evident from the characteristic yellowing of the foliage of affected trees. Figures 3(a) and 3(b) show the decline in chlorophyll content in both tree and twig experiments. The amount of soluble protein in needles was increased after mucus treatment [Figs. 3(c) and 3(d)] and, in addition, higher levels of activity of two enzymes, peroxidase and amylase, were noted. Peroxidase activity increased progressively from the second day after treatment right through to needle abscission [Figs. 3(e) and 3(f)]. Electrophoresis using polyacrylamide gels showed that nine anode-migrating and two very weak cathode-migrating peroxidases were present in the extracts (Fig. 10). Mucus treatment resulted in enhanced activities of all the regular peroxidases (except bands 1 and 2) and the appearance of two new peroxidases, bands 7 and 8. Amylase activity in needles of treated trees was increased (after a sharp initial fall) [Fig. 2(c)]. This increase in amylase activity (after day 7) was correlated with the decline in starch content ( $r = 0.75$ ), and was also reflected in an increased level of soluble sugars [Fig. 2(e)] appearing in the needle extracts. In experiments with twigs, on the other hand, any effect of mucus on amylase activity was very slight compared to that of twig removal, and no distinct correlation with starch or soluble sugar content was apparent.

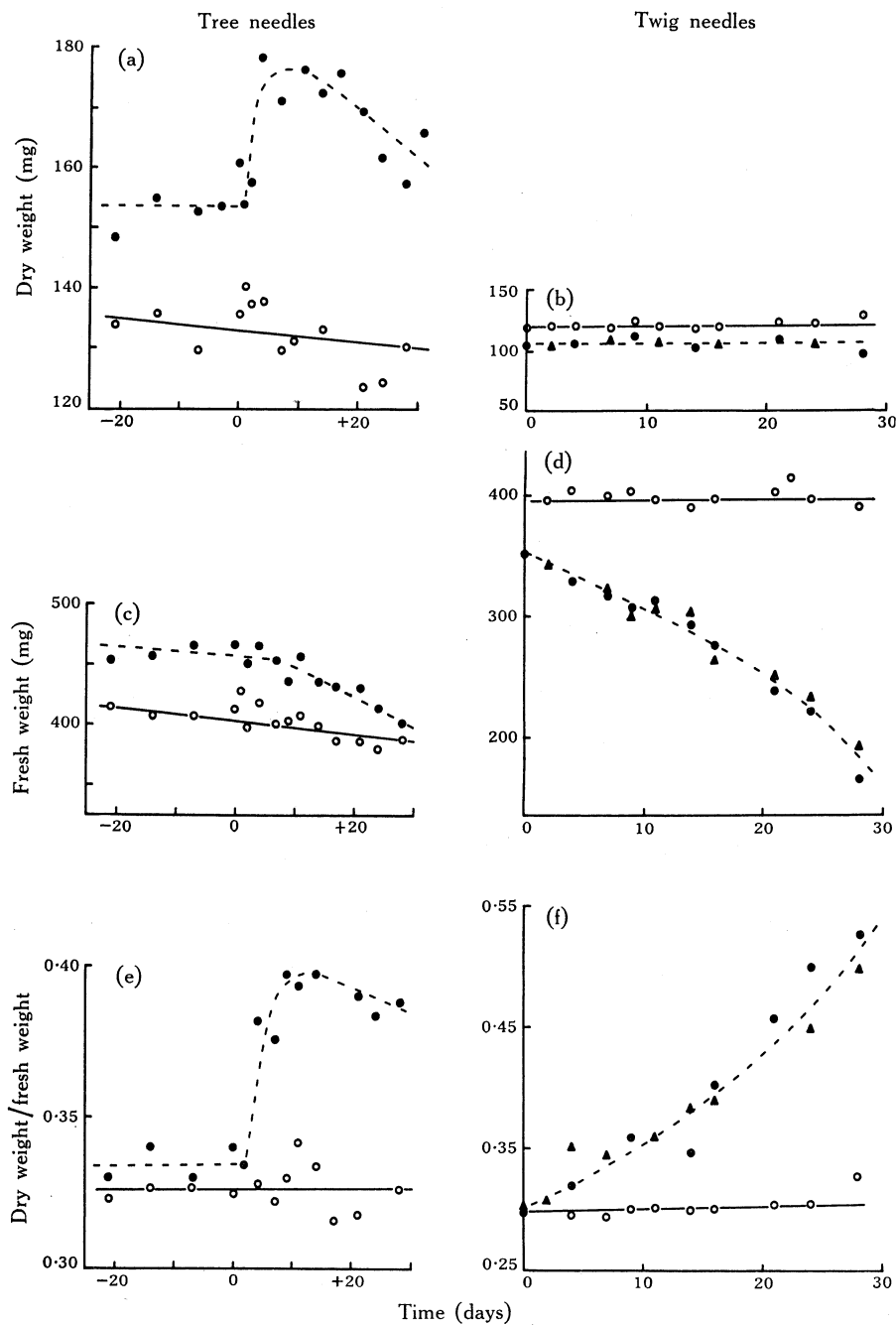


Fig. 1.—Effects of *S. noctilio* mucus on dry weight (a and b), fresh weight (c and d), and dry weight/fresh weight ratio (e and f) of *P. radiata* needle segments from intact trees (a, c, e) and isolated twigs (b, d, f). ○ Control. ● Treated with raw mucus. ▲ Treated with autoclaved mucus. *P* values are as follows:

		Days	Control	Raw mucus	Autoclaved mucus
Dry weight	Trees	—14-0, 4-14	0.02	<0.001	—
	Twigs	0-7, 16-28	0.5	0.8	0.3
Fresh weight	Trees	—14-0, 14-28	0.01	<0.001	—
	Twigs	0-7, 21-28	0.8	<0.001	0.001

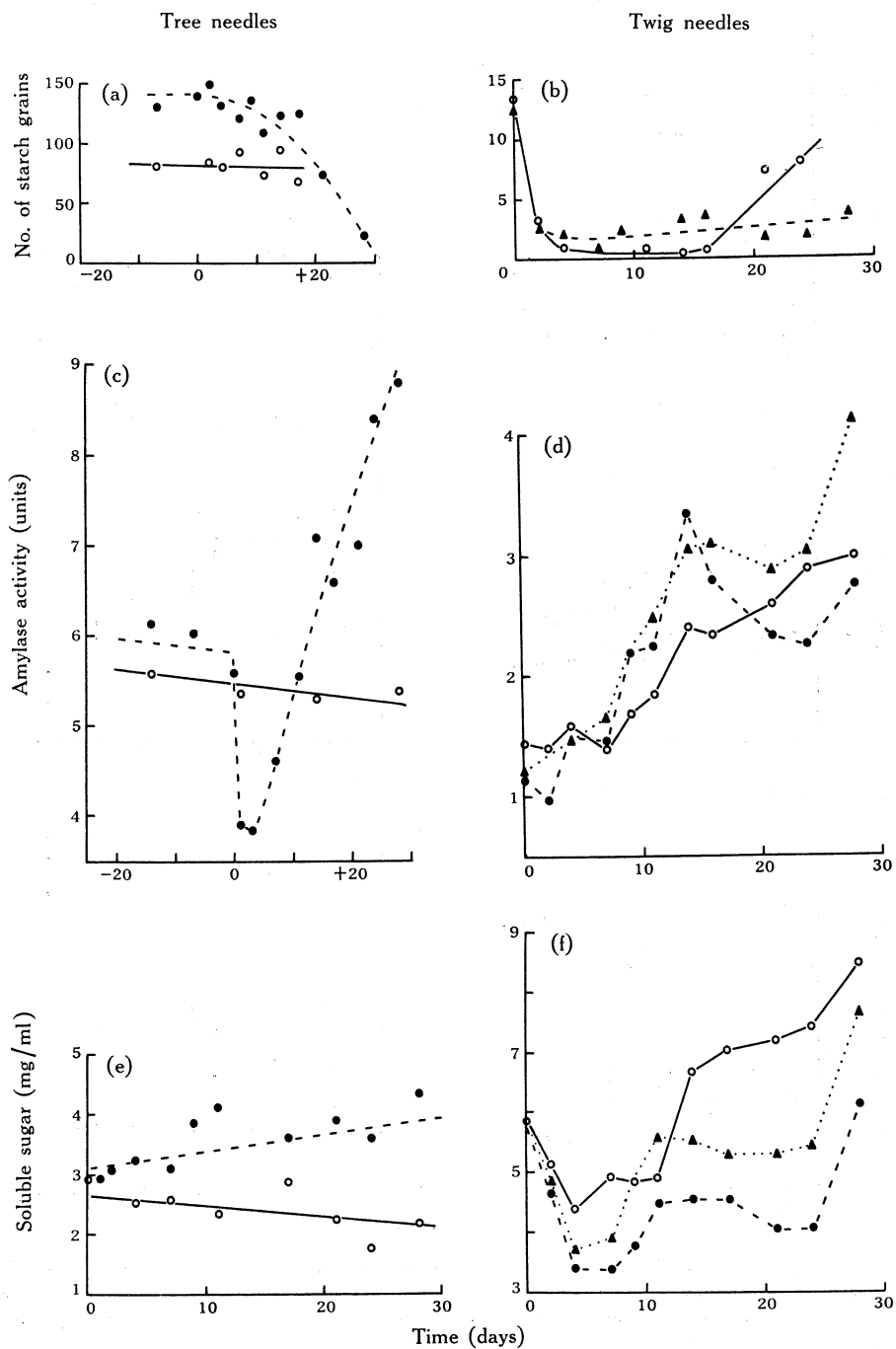


Fig. 2

Chromatographic examination of "free" sugars present in needle extracts showed no qualitative differences due to mucus treatment. The following sugars (with their relative amounts) were identified: glucose + + +, galactose + +, arabinose +, mannose +, ribose, trace amount only.

Another major effect of mucus treatment was a significant stimulation of respiratory activity. Madden (1968) has previously reported that attack by *S. noctilio* results in an enhanced rate of respiration of phloem tissue in the tree trunk. In the present experiments, we have shown that the rate of respiration of tree needles had risen to 2.5 times the control rate by day 9 (Fig. 4). The respiratory mode was also altered; the higher values for the respiratory quotient indicate the adoption of a more anaerobic mode of respiration ( $R.Q. = 1.37$  for treated needles at day 28, whereas for control needles it remained at approximately 1.0 throughout the experiment). Excision of twigs resulted in an initial sharp fall in respiratory activity. However, in the control twigs respiration rate subsequently recovered to near normal and by this time the effect of mucus on the respiration of treated twigs was apparent. Excision alone caused a drastic change in respiratory quotient of twig needles, and the change was exaggerated when coupled with mucus treatment. The altered respiratory rate, and the associated change in respiratory quotient, almost certainly bear on other parameters which were measured concurrently, e.g. starch and soluble sugar levels. Due to this interaction between the respiratory rate and the levels of potential substrates, it is difficult to interpret the significance of changes in these substances.

Coutts (1969*b*) has reported on the heat stability of the active principle in the mucus. Our data suggest that autoclaved mucus may be more effective than raw mucus in promoting the physiological responses. Thus, compared with raw mucus autoclaved mucus promotes higher levels of amylase activity and soluble sugars [Figs. 2(*d*) and 2(*f*)], advances the onset of chlorophyll disappearance by almost a week [Fig. 3(*b*)], and causes a greater departure of respiratory quotient from the control level [Fig. 4(*d*)]. Raw mucus caused a greater effect with peroxidase levels alone [Fig. 3(*f*)].

Coutts (1969*b*) also reported that the mucus was highly specific for *P. radiata*, and was without effect on *Populus* seedlings and *Eucalyptus* saplings. However, we found that when disks of radish leaf were floated on a solution of mucus (0.5 mg/ml) in the dark, they showed loss of chlorophyll and development of opaque areas within

Fig. 2.—Effects of *S. noctilio* mucus on starch (*a* and *b*), amylase activity (*c* and *d*), and soluble sugar (*e* and *f*) in *P. radiata* needle segments from intact trees (*a*, *c*, *e*) and isolated twigs (*b*, *d*, *f*). One unit of amylase activity = 2.85 mg maltose per millilitre extract in 5 min at 25°C. Soluble sugar estimated as milligrams glucose per millilitre extract. ○ Control. ● Treated with raw mucus. ▲ Treated with autoclaved mucus. *P* values are as follows:

		Days	Control	Raw mucus	Autoclaved mucus
Starch	Trees	—7-0, 21	0.2	0.05	—
	Twigs	2-9, 19-28	0.01	—	0.7
Amylase	Trees	—14-0, 1-4	0.2	0.01-0.001	—
	Trees	—14-0, 14-28	0.02	0.001	—
	Twigs	0-7, 14-16	<0.001	0.001	<0.001
Soluble sugar	Trees	0-4, 11-28	0.9	0.1	—
	Twigs	4-7, 14-16	0.02	<0.001	0.001

3 days (control disks showed loss of chlorophyll after 10 days), suggesting that *S. noctilio* mucus could accelerate senescence in this tissue. A similar result was obtained using needle segments of *P. radiata*. Addition of kinetin (0.01 mg/ml) inhibited the effect in both radish disks and *P. radiata* needle segments.

#### IV. DISCUSSION

Mucus from dissected glands of *S. noctilio* has been shown to induce a progressive series of changes in 1-yr-old needles of *P. radiata*, which may lead eventually to their death and abscission from the tree. The rapidity with which some of the symptoms appear (e.g. increase in respiratory activity within 24 hr, see Figure 4), shows that the active principle in the mucus is transported rapidly and efficiently from the site of application to the needles. The immediate effect of mucus appears to involve some impairment of the normal water relationships of the needles, which causes tissue desiccation, distortion, and eventual collapse of the translocatory cells of the phloem. Concurrent with these effects are some dramatic changes in the rates of certain metabolic activities, e.g. respiration, as well as changes in the levels of peroxidases and amylases. It is not possible at this stage to determine which of the total observed effects are primary, and which are of secondary importance. However, there is clearly a considerable degree of interaction of the effects, both in respect to the time of their appearance, and in their manifestation within the needle tissues.

In general our results support the observations reported earlier by Coutts (1969*b*). In one respect, however, there is an apparent anomaly which requires explanation. Coutts claimed that the increase in needle dry weight (trees) could be largely attributed to starch accumulation in the mesophyll cells. He further suggested, on the basis of shading experiments, that this starch was due to current photosynthesis, either by an increased rate of photosynthesis, or an impeded rate of translocation of photosynthate from the needles, or both. We did not observe starch accumulation in our experiments (Fig. 2). Moreover, the rapid loss of chlorophyll from mucus-affected needles (Fig. 3), and the failure of these needles (in experiments with twigs) to restore starch to the control level [Fig. 2(*b*)] are taken as evidence of lowered photosynthetic activity.

Fig. 3.—Effects of *S. noctilio* mucus on chlorophyll (*a* and *b*), soluble protein (*c* and *d*), and peroxidase (*e* and *f*) levels in *P. radiata* needle segments from intact trees (*a*, *c*, *e*) and isolated twigs (*b*, *d*, *f*). Chlorophyll content is expressed as milligrams per millilitre extract per gram dry tissue, soluble protein as milligrams equivalent to 1 ml bovine serum albumin, and peroxidase activity as change in optical density at 430 nm per minute per gram dry tissue. ○ Control. ● Treated with raw mucus. ▲ Treated with autoclaved mucus. *P* values are as follows:

		Days	Control	Raw mucus	Autoclaved mucus
Chlorophyll <i>a</i>	Trees	–21–0, 14–28	0.3	<0.001	—
Chlorophyll <i>b</i>	Trees	–21–0, 14–28	0.8	<0.001	—
Total chlorophyll	Twigs	0–4, 21–28	0.5	<0.001	<0.001
Protein	Trees	–14–2, 11–25	0.02	<0.001	—
	Twigs	0–2, 11–16	0.02	0.05	<0.001
Peroxidase	Trees	–21–0, 7–21	0.02	<0.001	—
	Twigs	0–2, 21–28	0.01	0.001	0.001



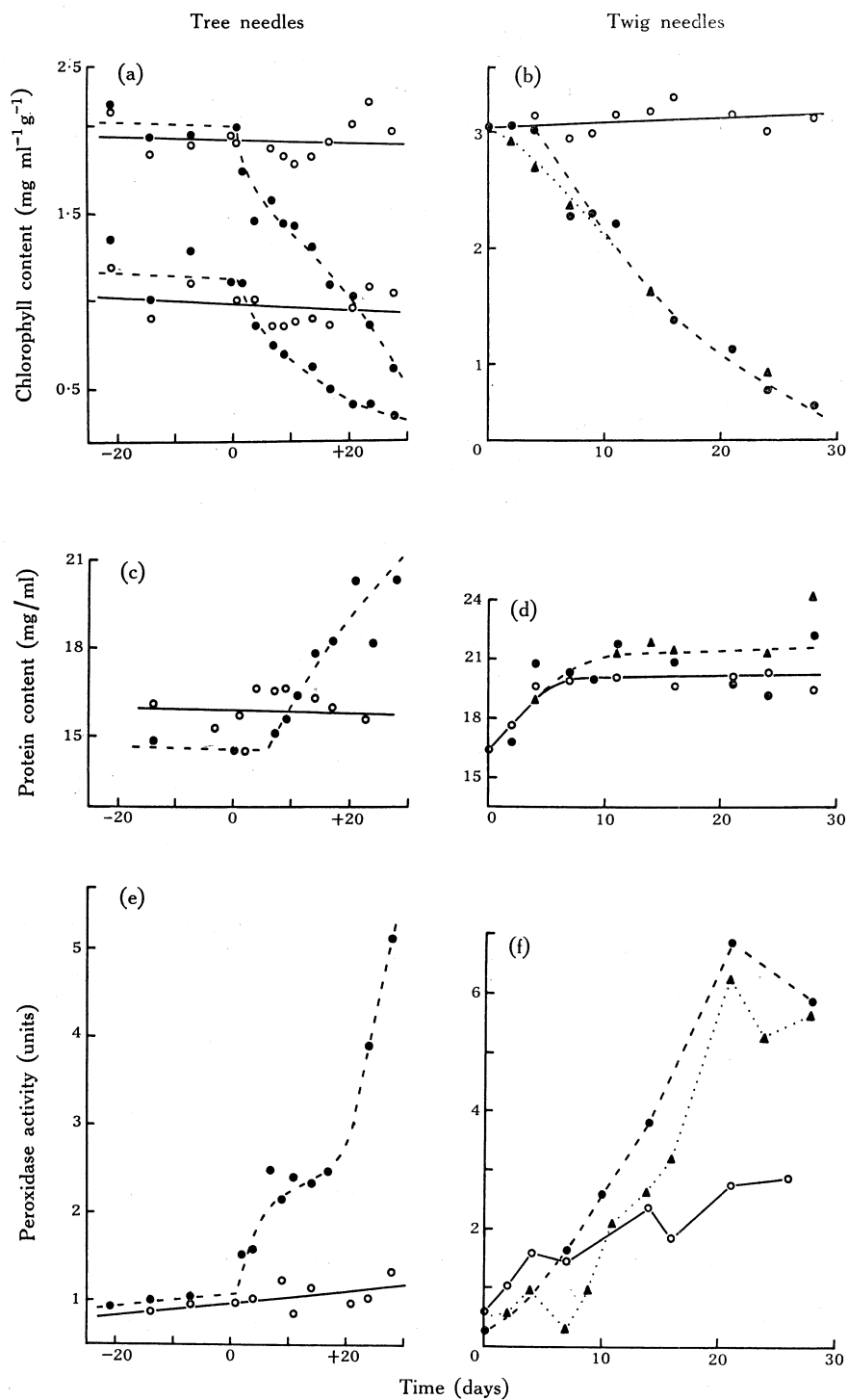


Fig. 3

Since conducting the present experiments, we have learned (personal communication from T. E. Bird) that Coutts did not observe starch increase in all trees injected with mucus. In general, only those trees that had some degree of resistance to *S. noctilio* attack showed positive accumulation of starch. However, our experiments

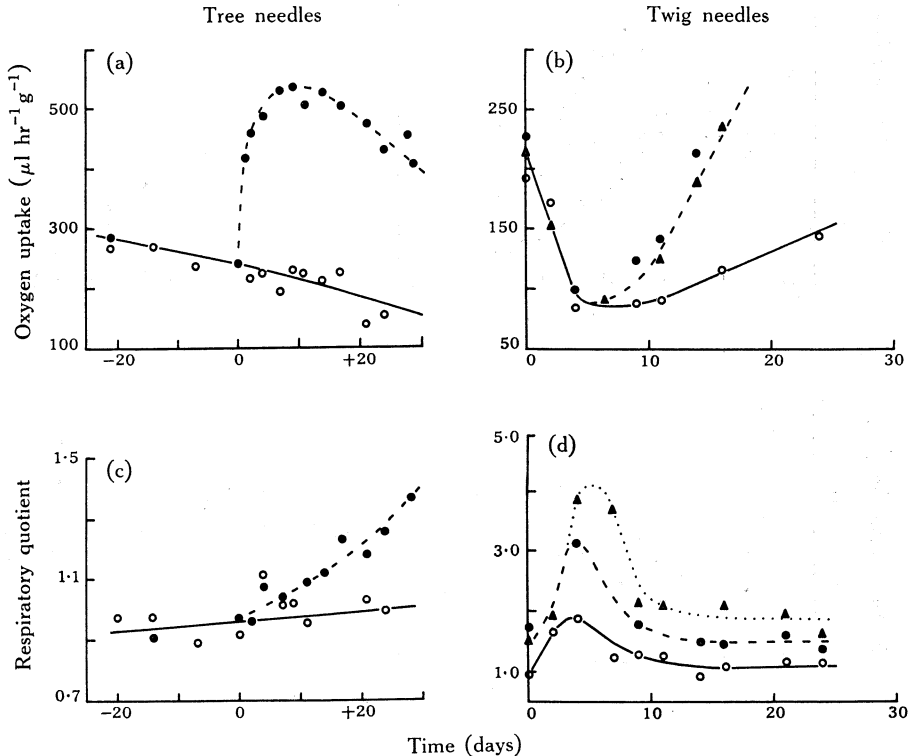


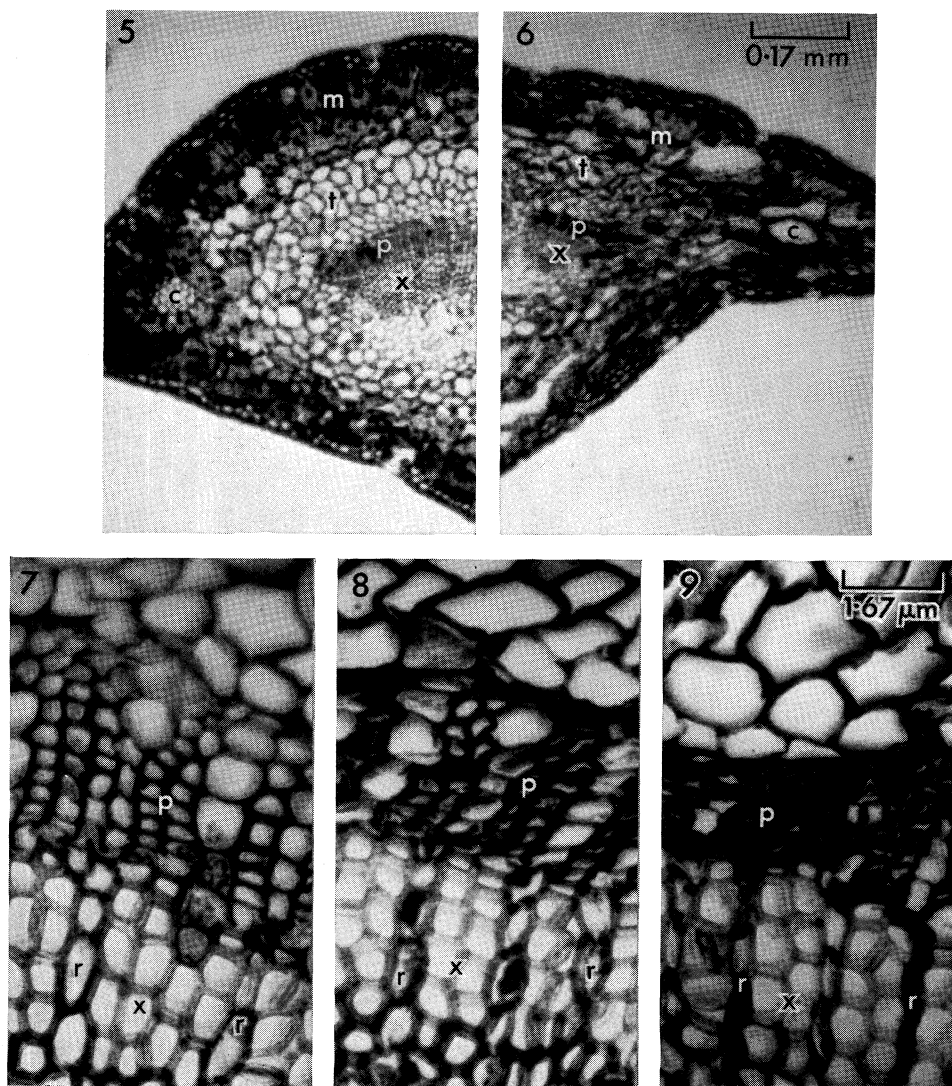
Fig. 4.—Effects of *S. noctilio* mucus on oxygen uptake (a and b) and respiratory quotient (c and d) of *P. radiata* needle segments from intact trees (a and c) and isolated twigs (b and d). ○ Control. ● Treated with raw mucus. ▲ Treated with autoclaved mucus. *P* values are as follows:

		Days	Control	Raw mucus	Autoclaved mucus
Oxygen uptake	Trees	−21–0, 7–17	0·01	<0·001	—
	Twigs	9–11, 16–21	0·05	0·01	0·02
Respiratory quotient	Trees	−21–0, 7–17	0·8	0·001	—
	Twigs	0–2, 4	0·2	0·01	0·01

were carried out on trees deliberately chosen for their high susceptibility to *S. noctilio*, in order to maximize the effects. It is clear that some additional work is necessary to determine the spectrum of physiological responses to mucus in trees of varying susceptibility. It is possible that such a study could provide information of potential use for field diagnosis of tree susceptibility, and may have application in future silvicultural practice.

As an alternative explanation to account for the increase in needle dry weight, we propose that mucus injection into the tree trunk may well stimulate a short-term

translocation of substances into the needles from other parts of the tree. This translocate may well be carbohydrate in nature, and may in fact serve as a precursor



Figs. 5-9.—Photomicrographs of traverse sections of *P. radiata* needle segments. 5, control. 6, from mucus-treated tree 7 days after injection. 7, tissues of vascular region in untreated needle. 8, tissues of vascular region 4 days after mucus treatment, showing distortion of phloem. 9, vascular tissues 7 days after treatment, showing extensive necrosis of phloem and ray parenchyma. *c*, resin canal; *m*, mesophyll; *p*, phloem; *r*, ray; *t*, transfusion tissue; *x*, xylem. Scale on Figure 6 applies to both Figures 5 and 6, scale on Figure 9 to Figures 7, 8, and 9.

for starch synthesis in resistant trees. The possibility of a similar translocation into needles on excised twigs is, of course, eliminated, and therefore no comparable dry

weight increase of twig needles could be expected [Fig. 1(b)]. The period during which inwards translocation may occur must necessarily be brief, because of the rapid deterioration of the phloem cells after mucus treatment.

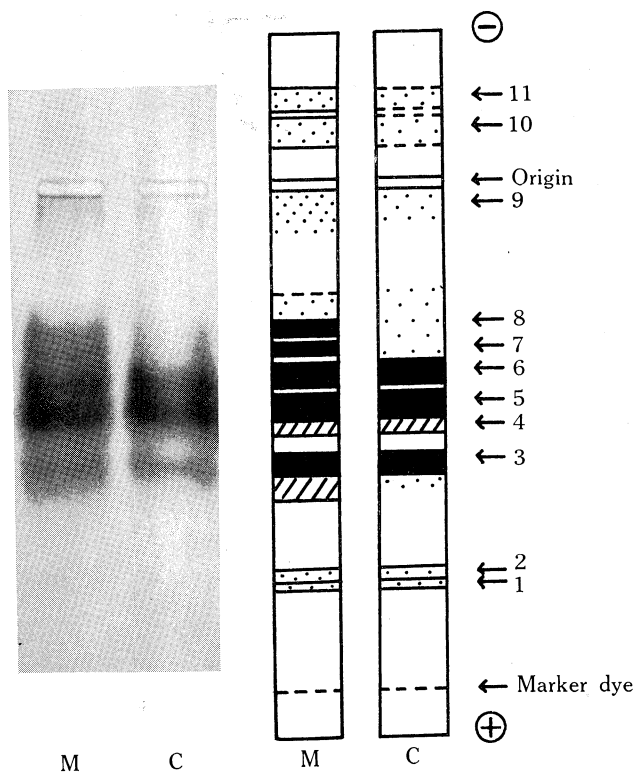


Fig. 10.—Photographs and interpretative drawings of acrylamide gel electrophoretogram, stained to detect peroxidase activity, of extracts from needle segments 7 days after injection of trees with mucus (M) or water (C).

In many respects the biochemical changes which *S. noctilio* mucus causes in *P. radiata* needles are similar to those which accompany the onset of senescence, either natural (Woolhouse 1967) or disease-induced, in the leaves of other plants. Thus Shaw and Samborski (1957), Collins and Scheffer (1958), Scott and Smillie (1966), and Németh and Klement (1967) have all reported stimulated respiratory activity in diseased plants, whilst Pethö (1966) noted higher levels of reducing sugars as well as increased respiration in maize leaves infected with crown gall. Doke and Hirai (1969) have reported increased amylase activity which was correlated with a stimulated rate, under dark conditions, of starch degradation in tobacco leaf disks infected with tobacco mosaic virus. Shaw and Colotelo (1961) observed an increase in the dry weight of susceptible rust-infected wheat leaves, accompanied by increase in the nitrogen content and level of free amino acids. Finally Weber *et al.* (1967), Lovrekovich *et al.* (1968), Jennings *et al.* (1969), and Curtis (1971) have all recorded increases in peroxidase activities in variously diseased plant tissues.

Variations from the control levels in each of these parameters are induced in the foliage of *P. radiata* by *S. noctilio* mucus. The affected needles are eventually abscised and shed. Thus it is possible to interpret these effects of mucus in terms of induced premature senescence. The reaction of the mucus in the standard senescence test using radish disks, and its reversal by kinetin, lend a measure of support to this notion. There is little knowledge yet about the chemical nature of the active principle in mucus, but the fact that it is quite stable at high temperatures suggests that it may be a substance of relatively low molecular weight.

Coutts (1969*b*) suggested that mucus may interact in some unknown way with natural growth hormones within the tree. On his interpretation, mucus acts essentially as a "tree-conditioning" agent which brings about a reduction in the effectiveness of the tree's natural resistance mechanisms. By this means, the successful establishment of the fungus is promoted and survival of the wasp's eggs and larvae is enhanced. The syndrome of responses in the affected needles, described in this paper, may express a type of hypersensitivity in which the tree, by premature senescence and shedding of its needles, attempts to remove the mucus toxin, thus increasing its chances of recovery. In this context hypersensitivity is synonymous with resistance. Field observations on trees attacked by *S. noctilio* indicate that those trees which shed their old needles soon after a moderate degree of attack often recover, but death usually results when the young needles are also affected. Young needles are more likely to be affected in the highly-susceptible trees.

#### V. ACKNOWLEDGMENTS

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