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Bacteria and Yeasts Associated with *Sirex noctilio*

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Five genera of bacteria and yeasts commonly occur in the oviposition drills and larval galleries of *Sirex noctilio*. In exceptional circumstances, some of these microorganisms may be pathogenic while the yeast, *Saccharomyces* sp., is involved in the production of attractants of the rhyssine parasitoids *Rhyssa* and *Megarhyssa* spp. A possible role in larval nutrition is suggested but these microorganisms have no role in the phytotoxic effects of *Sirex* attack.

INTRODUCTION

There are no previous records of pathogenic bacteria from siricid woodwasps (Hymenoptera:Siricidae) (Wolf, 1967). However, examinations of trees infested with *Sirex noctilio* have indicated that under certain conditions infection and death by bacteria may occur. Madden (1968) and Spradbery (1971) suggested that, in addition to the symbiotic fungus, *Amylostereum areolatum*, microorganisms could also be involved in the location of the siricid host by rhyssine parasites and Bedding (personal communication) considers that bacteria may influence the life cycle of the parasitic nematode *Deladenus siricidicola*.

The possibility also exists that bacteria and yeasts could affect insect nutrition and contribute to the pathogenic effects of *Sirex* attack on the host tree, *Pinus radiata*.

This paper reports findings of investigations on some of these possibilities.

MATERIALS AND METHODS

Billets infested with *S. noctilio* were obtained from eight localities in the State of Tasmania and from one site in Victoria. Sections of these billets were dissected and well-grown larvae removed. Small quantities of freshly exposed frass were removed with flamed forceps, transferred to stoppered vials containing 10 ml of sterile water, and shaken to disperse the compacted frass. The larvae were dissected under mercuric chloride (1:1000), the fore- and hindgut ligatured with cotton and the intact midgut removed through eight to ten changes of sterile water to a flamed microscope slide. The gut was punctured with a sterile needle and the contents emptied into two drops of sterile water. The mixture was immediately streaked onto nutrient agar and a complementary set of plates was streaked with the frass suspensions. Cultures on blood–agar plates were used to test for the presence of any nutritionally fastidious forms and Burk's nitrogen-free agar employed for nitrogen-fixing forms. Both inoculated nutrient agar and blood–agar plates were held in an anaerobic jar to detect any obligate anaerobes. All plates were incubated for a minimum of 48 hr at 36° C.

Pure cultures of resulting colonies were obtained by streaking onto plates and subculturing to nutrient broth. The pure cultures were then subjected to microscopical and standard microbiological tests (Breed et al., 1957; Skermann, 1967) to identify the isolated organisms.

The microorganisms associated with the adult alimentary system were obtained by similar procedures. Incrustations were removed from the anal opening of newly emerged females, macerated in sterile water, and the mixture streaked onto nutrient agar.

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These incrustations are formed by the drying of a small opaque droplet of liquid which is present at emergence.

Organisms in oviposition drill shafts and beneath the bark in attacked trees were isolated by the following procedure. A block of wood, 2.5 cm^3 containing oviposition drills was chiseled from a tree and with the bark intact was surface sterilized with absolute alcohol. The bark was removed, the cambial region scraped with a flamed scalpel, and scrapings were placed in sterile water tubes. The block was then split to expose the oviposition shafts and the scrapings from the lower half of each shaft were placed in separate sterile water tubes. The respective scrapings were incubated for 16 hr and then streaked onto nutrient agar.

The relative densities of microorganisms in the frass directly behind actively feeding larvae were assessed by a serial dilution–direct count technique (Allen, 1957). An exposed gallery was marked into 2.5-cm intervals and the frass in each interval removed, weighed, and placed in 10 ml of sterile water, shaken, and 1-ml aliquots of the resulting suspension serially diluted three to four times. A 1-ml aliquot of the final suspension was added to cover the surface of a nutrient agar plate. Visible colonies were counted after 48 hr incubation at 36°C and counts were related back to the original dry or fresh weight of the sample.

The pathogenicity of the isolated organisms with respect to the insect and the host tree was measured by direct injection of bacterial suspensions. For the insect pathogenicity tests, batches of 20 larvae and adults of known weight and size were injected with 2 μl of a standardized nutrient broth culture of each test organism. Control groups of insects were injected with sterile broth. The inoculated insects were held in separate vials and examined each day when the gross appearance, activity, and survival was noted. For possible pathogenic effects to the host tree, broth cultures were (1) inoculated into 30–40 drill holes (0.72 cm diam) made in intact branchlets of radiata pine and (2) twigs (30 cm long) were collected and stood in diluted broth cultures. These treatments were examined frequently and compared to untreated twigs for the presence of chlorosis or wilting.

The rhyssine parasites *Rhyssa persuasoria* and *Megarhyssa nortoni nortoni* will probe freshly exposed frass trails with their ovipositors and the same response is elicited when the frass is confined to glass vials stoppered with moist cotton plugs. Consequently, to test the effect of organisms on the attractiveness of frass, equal amounts of dried frass were placed in 10-ml glass vials, water added to saturation, stoppered, and autoclaved. The vials were then positioned in holes cut in a 4 × 4 design on a polyfoam board measuring 30 cm^2. Each vial was inoculated with a test organism and treatments were randomized. Rhyssine parasites were confined in 30 cm^3 cages and provided with honey and water. A sheet of paper was placed on the roof of the cages and the boards containing the vials inverted to bring the cotton stoppers in contact with the paper sheet. A small quantity of water was added to the vials each day to make up for evaporative losses and the sheet of paper was replaced. The position and the number of ovipositor punctures made in the paper was recorded. One percent mercuric chloride was added to control vials to suppress microbial growth. In a second experiment, frass was deleted from the vials and replaced with cotton plugs. Half of the vials were impregnated with yeast culture and the remainder with water. The probing response by the parasites was evaluated.

RESULTS

Two yeasts and six bacterial genera commonly occurred in the gut and frass of larvae collected from the billets of different localities. All forms were either aerobic or facultatively anaerobic. The identity of these organisms and their occurrences are summarized in Table 1. The two species of *Flavobacterium* differed in pigmentation, one (1) being highly coloured and the other (11)

TABLE 1
Organisms Isolated from Larvae and Frass of *Sirex noctilio* Collected from Billets from Nine Localities (1967–1970). (+) Present: (−) Absent.

Source		Organism							
		Saccharomyces	*Rhodotorula*	*Flavobacterium* 1	*Flavobacterium* 11	*Bacillus*	*Azotobacter*	*Acetobacter*	*Achromobacter*
Pittwater (T.)	Gut	−	−	+	−	+	−	−	−
	Frass	+	+	+	+	−	+	+	+
Claremont (T.)	Gut	−	−	+	−	−	−	−	−
	Frass	+	+	+	+	+	+	+	+
Blackwood Ck. (T.)	Gut	+	−	+	−	+	−	−	−
	Frass	+	+	+	−	+	+	+	−
Kettering (T.)	Gut	−	−	−	−	+	−	−	−
	Frass	+	−	+	−	−	+	+	−
Bruny Is. (T.)	Gut	−	−	−	−	−	−	−	−
	Frass	+	−	−	+	+	+	+	−
Bracknell (T.)	Gut	−	−	−	−	−	−	−	−
	Frass	+	−	+	−	+	+	−	−
Campania (T.)	Gut	−	−	−	−	−	−	−	−
	Frass	+	−	+	−	−	+	+	+
Brighton (T.)	Gut	−	−	−	−	−	−	−	−
	Frass	+	−	+	−	−	+	+	−
Scottsdale (T.)	Gut	−	−	−	−	−	−	−	−
	Frass	+	−	+	−	+	+	−	−
Victoria	Gut	−	−	−	−	−	−	−	+
	Frass	+	−	−	+	−	+	+	+

Material from Pittwater, Claremont, Blackwood Creek, and Kettering, obtained in fresh condition: others moderate to dry, many larvae had ceased to feed.

of dull luster. The latter form was not as commonly isolated. *Azotobacter* sp., present in frass from all localities, grew well on Burk's nitrogen-free agar. Microorganisms were always found in the gut when it contained particulate matter but in dry billets, larvae had ceased to feed and the gut contents had been voided. The occurrence of bacteria in these larvae was rare and their absence was achieved experimentally by placing active larvae onto Petri dishes for 48 hr during which time the particulate frass and its organisms were excreted.

There was an absence of molds in fresh frass although the blue stain fungus, *Ceratocystis* sp., did occur at low frequency. The absence of *Amylostereum* in the fresh frass must reflect the efficient extraction and utilization of the fungus from the wood ingested by the larvae. This fungus permeates the wood surrounding the larval gallery and ultimately reinvades the frass when its moisture content has fallen to much lower levels (100% oven dry wt). Conditions in the fresh frass strongly favor bacterial rather than fungal growth for moisture content is high (180% ODW) and aeration is low.

Few microorganisms occurred in the gut of adult insects but most forms were present in the anal incrustation. The occurrence of organisms in the incrustations of 25 newly emerged females is shown in Table 2. *Saccharomyces* sp. and *Flavobacterium* sp., alone or in combination, were the predominant organisms.

The cambial region of freshly attacked trees was essentially sterile but 24 hr after attack infection was high and *Saccharomyces* was the major form (29 occurrences in 34 tests). All ovipositions were contaminated with the symbiotic fungus and 36 of the 40 samples tested also contained *Saccharomyces* sp. Of the other samples, two contained *Bacillus* sp. and two contained *Sarcina* spp.

The actual density of organisms present in

TABLE 2
Organisms Recovered from Anal Incrustations of 25 Newly Emerged *Sirex noctilio*

Organism	Occurrence	Joint occurrences with *Saccharomyces* spp.
Saccharomyces	11	4
Flavobacterium	10	1
Bacillus	1	2
Serratia	3	1
Achromobacter	1	
Fungus (*Amylostereum* spp. *Ceratocystis* spp.)	8	

the frass behind actively feeding larvae varied within and between galleries but in all galleries sampled there were fewer organisms directly behind the larvae than in more distal samples. These differences are apparently related to the rate of colonization and the moisture content (and/or aeration) of the frass (Table 3).

All test organisms were pathogenic when directly injected into the insect hemocoel, and larvae were more susceptible than adults for a given density of inoculum, e.g., 50% of the *Sirex* larvae injected with 2 μl of a *Flavobacterium* suspension (ca. 10^8 organisms/ml) were moribund 2.5 days postinjection while it took 5.5 days for 50% of *Sirex* adults to succumb.

Dissection of moribund insects revealed a generalized septicemia which was more evident in larvae than in adult insects.

The organisms had no obvious effect on the host tree. Bacterial growth was favored in the diluted broth culture in which severed

TABLE 3
The Relative Density of Microorganisms/g Frass (ODW) and Moisture Content of Frass Removed from Five Galleries in the Same Infested Billet

	Distance behind larvae (cm)		
	0–2.5	2.5–5.0	5.0–7.5
No. organisms/g dry wt	2.7×10^4	4.1×10^4	12.2×10^4
Moisture content (%)	95	80	45

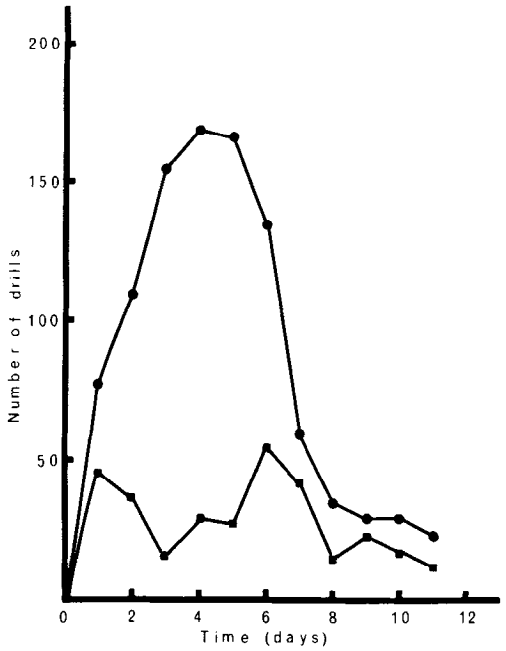

FIG. 1. Probing response of *Megarhyssa nortoni* females to sterile frass (■) and frass inoculated with *Saccharomyces* spp. (●).

branches had been placed, but there was no evidence of chlorosis or wilting.

Inoculation of sterile frass with organisms followed by bioassay with rhyssine parasites indicated that *Saccharomyces* enhanced the attractiveness of the frass to both insect species tested. The level of attractiveness increased in time from inoculation (Fig. 1) and after 5 days the *Saccharomyces* frass interaction was five to eight times more attractive than the sterile, control frass. The attractiveness of this yeast treatment then declined and relatively small increases in attractiveness occurred in the other treatments. A general contamination by yeasts had occurred at this time. Cotton impregnated with yeast suspension elicited only moderate probing by the parasites.

DISCUSSION

A general consistency in the kinds of organisms from the larval gut and frass trails of *S. noctilio* was found irrespective of the source of the material tested. This consistent relationship and the gradient of population density in the gallery indicated a mul-

tiplication of similar types in frass. The larval cavity is contaminated and excretions from the larvae favor this multiplication. The original contamination of the gallery occurs at the time of oviposition and it is possible that contamination of the ovipositor occurs as the adult emerges, for the anus and ovipositor sheath are in close association. *Achromobacter* is considered to be a rare contaminant.

The role of the microorganisms in larval nutrition is obscure. It has been reported (Brock, 1970) that lignin is utilized by *Flavobacterium* and ammonia by *Azotobacter*, while the provision of vitamins in insect diets by yeasts has been reported by Buchner (1965).

There was an absence of molds and no growth of *Amylostereum* from new frass indicating the efficient extraction of this material from the ingested wood. The physical characteristics of fresh frass also tended to exclude *Amylostereum*. Coutts (1965) reported that the optimal moisture content for growth of *Amylostereum* was approximately 70%.

It has been demonstrated that any of the organisms may be pathogenic when they directly enter the insect. Rupture of the gut or body wall within the gallery appears unlikely except following stinging by the rhyssine parasites. Not all stung larvae have eggs deposited on them so that ultimate death of these larvae may be brought about by bacterial infection. The pathogenic effect on the host tree following *Sirex* attack does not involve the bacteria and yeasts considered here. Recent work (Coutts, 1970a, b) indicates that it is the combined effects of the fungus and insect mucosecretion which kills the tree.

The regeneration of attractiveness of sterile frass by *Saccharomyces* and the temporal change in the insects response, which is similar to the changing attractiveness of sites on infested billets, strongly indicates the involvement of yeasts as a component in the host location behaviour of the rhyssine parasites. Though yeast alone was only weakly attractive to the parasites, its occurrence in frass does result in the production of materials to which parasites significantly respond.

Although the relationship between the microorganisms and *Sirex* is a passive one, the similarity in forms nevertheless indicate a regular succession, the effects of which may influence insect nutrition and host finding behavior of rhyssine parasites. Furthermore, under certain conditions, these forms may contribute to larval mortality.

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