FUNGAL ASSOCIATIONS IN THE BUILD-UP AND DECLINE OF *CRYPTOCOCCUS FAGISUGA* POPULATIONS[1]

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Abstract.--The fungal flora of *Cryptococcus fagisuga* colonies on *Fagus sylvatica* bark included the entomogenous species *Verticillium lecanii* wherever infestation was or had been very heavy. This fungus seemed to accelerate insect mortality *in vitro*. *Cladosporium cladosporioides* was present at all stages of insect colony development and, with other fungi, caused blackening of the wax secretion.

INTRODUCTION

The great economic importance of many species of scale insect has long made them a major object of man's efforts in biological control. Their colonial, sessile habit and predictable occurrence in space and time favours the use of predators and parasites. As the primary agent in the beech bark disease complex (Ehrlich 1934, Parker 1974) *Cryptococcus fagisuga* Lind. seems a worthwhile target for biocontrol, and yet it has been studied very little to this end.

Several predators of *C. fagisuga* are known. These include the coccinellids *Exochomus* and *Chilocorus* spp., the cecidomyid fly *Lestodiplosis* sp. and various chrysopids and hemerobiids, but their importance in regulating populations of the insect is thought to be limited (D. Wainhouse pers. comm.). No insect parasites of *C. fagisuga* are known.

As far as microbial control agents are concerned, only one fungus has been reported to exert some influence over *C. fagisuga*. This is *Ascodichaena rugosa* which forms a tough, black stroma at the bark surface, thus deterring the insertion of the insect's stylets (Houston 1976; Houston, Parker and Lonsdale 1979). No association between *C. fagisuga* and any entomogenous fungus has been reported, although it seems clear that a microflora is associated with the insect colonies, as evidenced by the commonly observed blackening of the white wax secretion. This blackening has been recognised as an indication that the colonies contain many dead insects and are probably declining. It appears that blackening is also related to the degree of wetting from rain, in relation both to water runnels on stems and to varying weather conditions.

The microflora of infested bark merits study not only for the possible detection of entomogenous fungi but also for elucidating the pre-infection ecology of bark-invading species of *Nectria*. It is of particular interest that *C. fagisuga* is consistently associated with *Nectria coccinea* Fries. or with *N. coccinea* var. *faginata* Lohman, Watson & Ayers in the beech bark disease complex. Some *Nectria* spp. are known as parasites of scale insects (Petch 1921) and although this does not necessarily imply that the relationship between *C. fagisuga* and *N. coccinea* involves parasitism, it does seem likely that *N. coccinea* is ecologically favoured within the microflora of *Cryptococcus*-infested beech bark (Lonsdale and Sherriff, these Proc.).

The objectives of the present work were to investigate the fungal flora associated with different phases in the growth and decline of *C. fagisuga* populations and to examine the possibility that the insect is susceptible to attack by entomogenous fungi.

EXPERIMENTAL

Detection of fungi associated with *C. fagisuga*

Throughout this work several types of *C. fagisuga* colony development were defined so as to represent different phases of build-up and decline. In defining these types, the colonies were classified by size and by the extent to

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which dead insect bodies and blackened wax had accumulated. In this way, colonies are described as small or large and new or old. Where appropriate, a score class for the overall insect density on the stems was used in combination with the classification of colony type.

Preliminary observations indicated that the most frequently occurring fungi on *C. fagisuga*-infested bark were present at all forest sites studied, and that all heavily infested trees carried populations of these fungi. Three fungi commonly isolated were *Verticillium lecanii* Viegas which is an entomogenous species (Petch 1948), a synnematous *Acremonium* (the imperfect stage of *Nectria viridescens* Booth) and *Cladosporium cladosporioides* (Fres.) de Vries which seemed primarily responsible for blackening of the wax secretion.

On the basis of these observations, a series of platings of individual live second instars was set up on 0.01% YEA. These insects were obtained from trees at Alice Holt Forest, Hampshire and were removed by dissection of colonies *in situ*. Five different types of insect infestation were recognised in this survey as shown in Table 1, which includes all fungi which were isolated from 10% or more of insects in at least one of the infestation types. These data show that *V. lecanii* was abundantly present on insects where the infestation either was or had been in the form of extensive cover on the bark. *Nectria viridescens* and *C. cladosporioides* were present on insects from all infestation types. *Fusarium lateritium* Nees. seemed to be associated with old, declined infestations, while *Mucor* sp. was largely confined to small, recently established colonies. The fungi which occurred on fewer than 10% of the insects in

Table 1.--Fungi frequently isolated from live *C. fagisuga* adults and larvae plated on 0.01% YEA[b] (see text for other species)

Fungus	Percentage of insects yielding each fungus				
	A[a] (discrete)	B (discrete)	C (residual)	D (whitewashed)	E (residual) ('BBD tree')
Verticillium lecanii	0	0	56	69	74
Nectria viridescens	31	21	8	3	14
Cladosporium spp.[c]	25	36	16	3	6
Fusarium lateritium	0	0	16	0	9
Mucor sp.	38	0	4	0	0
Penicillium sp.	6	0	4	19	3

a: fuller descriptions of infestation types are: A, small isolated (≤2mm mean diameter), well separated colonies with little or no black wax; B, large (>5mm mean diameter), well separated colonies; C, low current infestation on trees with previously heavy infestation; D, stem continuously colonised over large areas; E, infestation pattern as for 'C' but bark with inactive, callused *Nectria* lesions.

b: yeast extract agar.

c: almost entirely *C. cladosporioides*.

any infestation type included the following hyphomycetes: *Trichoderma viride*, *Trichothecium* sp., *Fusarium avenaceum*, *Ramichloridium subulatum*, *Alternaria alternata*, *Stemphylium botryosum*, *Catinula* sp., *Diplodina* sp., *Pyrenochaeta* sp. (an un-named species), *Epicoccum* sp. *Monilia* sp. *Stachylidium* sp. *Dactyella* sp. and at least three non-sporulating fungi. Yeasts, especially *Aureobasidium* sp., were frequently present on insects from all colony types other than 'whitewashed', and bacteria were present throughout the samples. *Nectria coccinea* was not detected on individual insects, but was isolated from mass platings of insects together with their wax.

Of the fungi detected, *V. lecanii* was the only species known to include entomogenous strains, and a further study was carried out to gain further information on the extent to which this fungus was dependent on insect density. This study involved the counting of insect colonies from which *V. lecanii* grew during the incubation of 25mm discs of *C. fagisuga*-infested bark. The fungus was readily detectable after three or four days' incubation under humid conditions at room temperature. Only *V. lecanii* was scored in this way, although some of the fungi named above were also observed by this method, together with several *Acremonium* spp. and a *Sporothrix* sp. The discs were taken from trees with different overall infestation densities at Queen Elizabeth and Marden Forests in Hampshire and West Sussex respectively. The data, which are shown in Table 2, are based on infestation classes (scores 0 to 5) of the type used by Houston, Parker and Lonsdale (1979), classes 0 and 1 being excluded from the present study. Within each of these classes variation occurred in the apparent age of the colonies, and a classification of old and new infestation was used. 'Old' infestations were recognised by the presence of substantial blackening and erosion of the wax.

These bark disc incubations provided confirmation that *V. lecanii* was dependent on insect density and they also showed an effect of age of infestation. On new infestations, only class 5 yielded a substantial score for *V. lecanii*. However, in older infestations

Table 2.--Outgrowth of *V. lecanii* from incubated bark discs in July 1982

Infestation class		Mean percentage of individual wax masses yielding *V. lecanii*			
		2(light)	3(moderate)	4(heavy)	5(very heavy)
Colony type					
'New'	small[a] colonies	6	1	2	50
infestation	large[b] colonies	Abs.	0	0	100
'Old'	small[a] colonies	0	40	16	76
infestation	large[b] colonies	Abs.	29	73	94

a <3mm mean diameter b ⩾3mm mean diameter.

Abs. indicates large colonies absent in class 2 infestation.

For clarity, the above data are pooled for the two forest sites and expressed as percentages. Due to heterogeneity between sites, statistical significance, based on χ^2 tests of the actual counts was estimated separately for each site and for old and new infestation with the omission of poorly replicated classes. The effect of infestation class was highly significant ($p < 0.01$ to $p < 0.001$) except for small colonies on new infestations at Marden Forest ($0.10 < p > 0.05$).

the fungus was substantially present on colonies with a score class of three to four. This occurred whether or not most of the old colony material had been washed away by rain.

Plate 1.-- Conidiophores of *V. lecanii* growing from *C. fagisuga* colonies (x 100)

Fungal inoculations of *C. fagisuga in vitro*

In all the inoculation studies, individual live first instars were placed on sterilised filter paper in humidity chambers (95% R.H.) at 20°C. The inocula were applied as spore suspensions of equal concentration for each fungus, these being pipetted on to each insect (or in one trial, applied to the filter paper). The fungi were divided into two groups: species found on *Cryptococcus*-infested beech bark, and species of known entomogenous character which had been isolated in separate studies from beech wood but not from bark.

A satisfactory *in vitro* culture technique for the insects was not available and, for this reason, substantial mortality occurred in distilled water controls. The effects of the inoculations were assessed by comparison with these controls. After seven days' incubation, an excess of mortality compared with the controls seemed to occur with some of the inoculations, especially those involving *V. lecanii* isolates NDl and CFl (Table 3), but the differences were not significant. The natural fungal inocula carried by the insects included *V. lecanii* and *N. viridescens*, a factor which would have reduced any effect of artificial inoculation with these species.

In view of the difficulties in assessing the effects of inoculations on mortality, assessments for the beech wood fungi were carried out simply by scoring the production of external growth of the fungi on the insect bodies after death (Table 4). These date show that *Paecilomyces farinosus* was by far the most effective colonist of the insects.

Table 3.--Inoculations of *C. fagisuga* with bark fungi

Inoculant fungus	Insect mortality (excess % over control) 4 days	7 days	% isolation of fungus on 0.1% YEA Inoculated	Uninoculated
V. lecanii F3	0	+ 3	93	19
V. lecanii NDl	0	+17	70	19
V. lecanii CFl	0	+17	97	19
C. cladosporioides CFW	-6	-14	67	0
Nectria coccinea ACR	+4	+ 7	23	0
N. coccinea v. *faginata* 33A	0	+10	7	0
N. viridescens	-2	- 7	17	20
N. ditissima	NT	NT	0	0

NT signifies not tested. χ^2 tests, carried out on the counts of insects observed dead and alive, showed no significant effects of fungal inoculations at the 5% level.

Table 4.--Inoculation of C. fagisuga with potential entomogenous fungi obtained from beech wood

Inoculant fungus	% of insects giving rise to external growth of test fungi after death Inoculated	Uninoculated	No. in sample
Paecilomyces farinosus	80	0	20
Beauveria bassiana	0	0	20
Nodulisporium sp.	17	0	35
Isaria umbrina	0	0	22

Observations of individual insects inoculated with *N. coccinea* suggested that it failed to colonise them in the presence of a natural inoculum of *V. lecanii*. In view of the apparent mycoparasitic ability of *V. lecanii* (Lonsdale and Sherriff, these Proc.), a series of mixed inoculations was set up, with three different ratios of conidial concentration of the two fungi. The results showed a very high incidence (80%) of natural infection with *V. lecanii*, and this prevented a quantitative assessment of any competition between the fungi. However, it was found that *N. coccinea* was recoverable from insects inoculated with both fungi, even at a *Verticillium:Nectria* ratio of 3:1.

DISCUSSION

The main finding in the present work was that *Cryptococcus fagisuga* colonies carry a microflora which includes at least one fungus of an entomogenous type: *Verticillium lecanii*. This fungus showed a dependence on high insect density, or more precisely on the presence of substantial coalescence between insect colonies. Its absence in small isolated colonies of apparently recent establishment and its presence in older colonies of only moderate density, suggests a possible sequence of events in its colonisation. This sequence may begin with the spread of the fungus through the colonies when they reach an advanced stage of coalescence. Later, as the infestation declines, the fungus may persist, even though the population of the insect has perhaps become quite low. Such a relationship would be consistent with the apparent absence of aerially dispersed spores in this fungus and with its tendency to produce long conidiophores which trail over the insect colonies. Initial infection of young colonies could be effected through vector activity by micro-arthropods, a process observed during the present work.

It is not clear to what extent if any the decline of the insect population is due to infection by *V. lecanii*, and this uncertainty stems partly from difficulties of obtaining reliable results from inoculation experiments with *C. fagisuga*. However, the frequently recorded role of the fungus as a weak parasite of insects together with observations of rapid death of *C. fagisuga* crawlers in contact with heavy inocula, suggest that further studies are worthwhile.

None of the other fungi observed on *C. fagisuga* is of known insect-parasitic ability, but they are clearly of considerable interest in the ecology of *N. coccinea* (Lonsdale and Sherriff, these Proc.). It is interesting that *N. viridescens*, a frequently occurring member of the insect colony microflora, has a synnematous imperfect stage, since this sporing habit is common to many entomogenous fungi. Preliminary observations suggest that this and some other fungi may develop on dead insect bodies subsequent to the outgrowth of *V. lecanii*. It was not evident that *N. coccinea* develops in this way; indeed the apparent absence of this fungus on individual insects seems to confirm the findings of Stone (1967) who attempted to isolate *N. coccinea* var. *faginata* from *C. fagisuga*.

The dominance of *Cladosporium cladosporioides* on old insect colonies exemplifies the well known relationship between dematiaceous hyphomycetes and scale insects and aphids, although in most such cases the fungi are thought to utilise honeydew, an excretion which seems not to be produced by *C. fagisuga*. There is no reason to suppose that *C. cladosporioides* can parasitise the insect, but by dominating the microflora it could perhaps influence the development of entomogenous species. This may operate through competition for nutrients or through a reduction in the water-repellent properties of the wax secretion.

It remains unclear as to whether biocontrol of the insect is feasible. If it can be reliably shown that *V. lecanii* is capable of causing substantial mortality, it may be possible to introduce it at an earlier stage of insect build-up than seems naturally to occur. The fungus has been successfully used against aphids such as *Macrosiphonella sanborni* (Hall 1975) and scale insects such as *Coccus hesperidum* (Samšiňáková and Kálalová 1975). The apparent dual role of *V. lecanii* as a control agent for insect pests and fungal pathogens is an interesting possibility, first suggested by Hall (1980).

Manipulation of the bark surface environment may encourage development of *V. lecanii* from natural inocula; in particular the application of wetting agents to infested bark is a possible treatment suggested by the microbial overgrowth and decline of insect colonies in naturally wet conditions (D. Lonsdale unpublished data). The use of other fungi such as *Paecilomyces farinosus*, which readily colonised *C. fagisuga* bodies in the present work, also deserves further attention.

It remains unclear whether the microflora associated with *C. fagisuga* exerts an important control on populations of the insect. It seems likely, however, that the composition of the microflora may be an indicator of the state of insect build-up and decline on individual trees and within entire stands.

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RÉSUMÉ

La flore fongique associée aux colonies de C. fagisuga a été étudiée par mises en cultures et par observations directes. Les champignons Dématiacées, surtout Cladosporium cladosporioides, étaient présents à tous moments de la gradation de l'insecte et étaient responsables du noircissement de la sécrétion de cire des insectes.

Lorsque les colonies étaient devenues très grandes ou coalescentes, un champignon entomophyte connu, Verticillium lecanii, était généralement présent. Il était également présent dans les populations en déclin, même quand la densité des insectes était beaucoup réduite. Par contre, une espèce de Mucor était principalement limitée à de petites colonies isolées, tandis que le Nectria viridescens était présent pendant toutes les phases de développement des colonies. Le Fusarium lateritium était principalement associé aux populations en post-déclin.

L'inoculation in vitro de C. fagisuga avec plusieurs champignons a démontré que les corps des insectes sont facilement envahis par V. lecanii, N. viridescens, N. coccinea, C. cladosporioides et Paecilomyces farinosus. Il n'a pas été possible de déterminer de manière fiable l'effet des inoculations sur la mortalité des insectes, mais une augmentation non significative de la mortalité a été associée à V. lecanii.

ZUSAMMENFASSUNG

Die Pilzflora in Kolonien von C. fagisuga wurde anhand von Isolierungen und direkten Beobachtungen untersucht. Hyphomyceten mit dunklem Myzel, in erster Linie Cladosporium cladosporioides, waren in allen Entwicklungsstadien der Lauskolonien bis zu deren Zusammenbruch festzustellen und sind für die dunkle Verfärung der Wachswolle verantwortlich.
In sehr großen oder flächig ausgebildeten Kolonien war gewöhnlich Verticillium lecanii, ein von Insekten bekannter Pilz, anzutreffen. Dieser war auch in absterbenden und weitgehend abgestorbenen Kolonien vorhanden. Im Gegensatz dazu war eine Mucor-Art hauptsächlich auf kleine, isolierte Lauskolonien beschränkt. Nectria viridescens war hingegen in Kolonien aller Entwicklungsstadien zu finden. Fusarium lateritium trat hauptsächlich nach dem Zusammenbruch der Populationen auf.

Inokulationen von C. fagisuga mit mehreren Pilzen in vitro zeigten, daß die Insekten leicht von V. lecanii, N. viridescens, N. coccinea, C. cladosporioides und Paecilomyces farinosus besiedelt werden können. Die Wirkung der Pilze auf die Sterblichkeit der Insekten war nicht zuverlässig zu ermitteln, aber im Zusammenhang mit V. lecanii war ein, wenn auch nicht signifikanter Anstieg der Sterberate zu beobachten.

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