

LETTER TO THE EDITOR

Evidence for hyperparasitism of coffee rust (*Hemileia vastatrix*) by the entomogenous fungus, *Lecanicillium lecanii*, through a complex ecological web

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The entomogenous fungus, *Lecanicillium lecanii* is hyperparasitic on *Hemileia vastatrix*, the cause of coffee leaf rust in the laboratory, and has frequently been observed attacking it in the field. The existence of a complex ecological web involving the spatially clustered mutualism of an ant (*Azteca instabilis*) with a scale insect (*Coccus viridis*), where the scale insect was infected by *L. lecanii*, prompted a search for a spatial correlation between the attack of *L. lecanii* on the scale insect and the incidence of rust in a commercial coffee crop. A weak but statistically significant effect of hyperparasitic control of coffee rust was observed on two distinct scales: in a 45-ha plot and on a scale of approximately 10 m. It was concluded that this effect was linked to an indirect effect of the ant–coccid mutualism, where *L. lecanii* was a parasite of the coccid.

Keywords: *Azteca instabilis*, biological control, *Coccus viridis*, *Coffea arabica*, mutualism, spatial analysis

Introduction

Historically, coffee leaf rust, caused by *Hemileia vastatrix*, has been a devastating disease the world over, the main cause of abandonment of coffee as a commercial crop in Sri Lanka (then Ceylon), Sumatra and Java, as well as causing panic among producers throughout Central America in the 1980s. As it arrived in the Americas through Brazil, producers aware of its devastating consequences replanted what were held to be resistant cultivars, and reduced or eliminated shade cover, in the hope of curtailing its feared effects. Here it is hypothesized that the disease has antagonists that have acted to reduce its effectiveness in recent years and that those antagonists may be difficult to see directly because they operate through complex ecological relationships, as postulated previously (Avelino *et al.*, 2004). Evidence is presented that the entomogenous fungus *Lecanicillium lecanii* may be such an antagonist of *H. vastatrix*, operating through a spatially structured ecological network that includes a key ant–insect mutualism which allows for a peculiar spatial structure of this hypothesized antagonist.

While the coffee rust disease remains a major threat to producers all over the world, it has largely become more of an irritant than a disaster, with farms throughout Latin America showing low levels of infection almost every year. Phytosanitary methods, such as heavy pruning of shade trees, use of fungicides and planting of resistant cultivars, seem to have worked to some degree (Fulton, 1984), but even farms where dense shade cover has been retained and rust-resistant cultivars have not been planted have not suffered as severely as feared (McCook, 2006). It is possible that this reduced effect of the disease is partly caused by antagonists or natural enemies and, coupled with the lowered regional infection rate induced by large-scale planting of resistant varieties, has held this disease in relative check (Avelino *et al.*, 2004).

The importance of natural enemies in the phylloplane has been emphasized by several authors (Blakeman & Fokkema, 1982; Kushalappa & Eskes, 1989; Andrews, 1992). One of those enemies may be the hyperparasite, *L. lecanii* [previously known as *Cephalosporium lecanii*, part of what was previously identified as the *Verticillium lecanii* species complex (Kouvelis *et al.*, 1999; Zare & Gams, 2001)], known to colonize *H. vastatrix* under laboratory conditions (Eskes *et al.*, 1991) and observed attacking it in the field (Shaw, 1987; personal observations). Variability for infectivity exists among different

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isolates, ranging from 0 to 80% in tests on the whitefly *Bemisia tabaci* (Mor *et al.*, 1996). In the present research, because of an unusual spatial arrangement and a complex web of interactions in a large coffee plantation in Mexico, it was possible to test the hypothesis that *L. lecanii* contributes to the control of *H. vastatrix* under normal conditions of coffee production.

The particular circumstance that allowed this test was the existence of a spatially extended mutualistic system of an ant, *Azteca instabilis* (Hymenoptera, Formicidae), and the green coffee scale, *Coccus viridis* (Hemiptera, Coccidae) mutualist (Vandermeer & Perfecto, 2006). The ant constructs nests in shade trees of coffee plantations and is distributed in a non-random fashion, forming clusters of satellite nests in local groups of trees (Vandermeer *et al.*, 2008). *Coccus viridis* occurs throughout the plantation, but never forms large concentrations unless tended by ants, and almost always within clusters of nests of *A. instabilis*. *Coccus viridis* commonly contracts a disease, white halo fungus disease, caused by *L. lecanii*. This insect–fungal disease system is scattered around the coffee farm, but is rarely observed outside of the patches of *A. instabilis* colonies, probably because the scale insect normally escapes the disease as a result of its low local population density. Only when under the influence of the ant does the *C. viridis* population reach a population density high enough to permit *L. lecanii* to become epizootic. Additionally, on many occasions during the early summer of 2008, what appeared to be *L. lecanii* lesions were observed on the surface of *H. vastatrix* uredinia (rust pustules), but only in the vicinity of concentrations of *C. viridis* under attack by *L. lecanii*.

This paper postulates that this ant–hemipteran mutualism provides centres of concentrated production of spores of *L. lecanii* and thus contributes to an elevated level of control over *H. vastatrix*. If this is the case, it should be possible to detect a local spatial effect of the incidence of *H. vastatrix* with regard to the spatial distribution of the ant–hemipteran mutualism.

Methods

System

The study was conducted at Finca Irlanda, a 300-ha certified organic coffee farm in the Soconusco region of Chiapas, Mexico (15°11'N, 92°20'W), established over 100 years ago. The farm is typical of the general ecosystem of highland coffee production in Mesoamerica. The area is located 900–1150 m a.s.l. and receives *c.* 4500 mm rain annually, unevenly distributed in the rainy season (May to November) followed by a dry season (December to April). During the present study rainfall was not atypical for the region. In this region, coffee production is accomplished using a variety of techniques, but most often using some sort of shade tree, affording an understorey environment for the coffee bushes (Perfecto *et al.*, 1996; Perfecto & Armbrrecht, 2003). The farm under study has over 90 species of shade trees, about 70% of which are represented by

four species of *Inga* (Perfecto & Vandermeer, 2008), scattered in a uniform pattern across the farm's landscape (Vandermeer *et al.*, 2008) and casting an average of 75% shade in a relatively uniform manner throughout the farm. Consequently, the moisture regime at the level of the coffee bushes appears quite constant over the space occupied by the farm. The farm is classified as a commercial polyculture (Moguel & Toledo, 1999) and has, according to its owner, been in this state since its foundation. All coffee bushes are *Coffea arabica*, cvs Typica and Bourbon, with some plants grafted onto *Coffea canephora* rootstocks.

In 2004, a 45-ha permanent plot was established in which a total of 10 605 individual shade trees were marked and measured, involving 90 species. Several ant species nest in these shade trees, but by far the most evident is *A. instabilis*. Within a clump (group of *Azteca*-occupied shade trees), there is a unique food-web structure that has been qualitatively characterized as follows. The ants, which nest in the shade trees and only rarely nest in the coffee bushes themselves, tend scale insects (mainly *C. viridis*) on nearby coffee bushes (Vandermeer & Perfecto, 2006). Parasitic wasps (mainly two species of Encyrtidae) and a coccinellid beetle (*Azya orbiger*) routinely attack the scales, and reduce their local population density to almost zero in the absence of ants. The coccinellid beetle is myrmecophilous (having a tendency to be associated with ants) and is thus difficult to encounter except in the vicinity of the *Azteca* colonies (Liere & Perfecto, 2008). A parasitic fly (*Pseudacteon* sp., Phoridae) attacks the *Azteca* ants, presumably reducing their numbers, and demonstrably reducing their foraging efficiency (Philpott *et al.*, 2004), thus potentially restricting the expansion of the ant colonies.

The scale insects are routinely attacked by the white halo fungus, *L. lecanii*, but only when their populations are locally large, which only happens when they are under protection from the ants. In 7 years of research on this system, *L. lecanii* has only rarely been encountered not associated with the ant–scale mutualism, and then only on leaves and berries with especially large concentrations of scales.

Coffee rust, caused by *H. vastatrix*, is endemic in the region and is regarded by producers as a constant threat. According to the producer, rust is present, but seems to only reach important pest status in relatively small (less than 1 ha) patches on the farm and very irregularly from year to year.

Field surveys

Surveys were done on two different scales, one over the entire 45-ha permanent plot, and four smaller, more detailed surveys. At the large scale, a 50- × 50-m grid was imposed over the whole 45-ha plot, with a shade tree located near the centre of each grid square. If *A. instabilis* nests were extant in the grid, a tree containing a nest was used as the centre, otherwise, the tree nearest the centre of the 50- × 50-m plot was used as a reference. The five

coffee bushes nearest the reference tree were sampled using the standard technique of a quasi-random harvest of 10 leaves from each bush (taken from the top, middle and bottom thirds of the bush) and recording how many of the 10 leaves exhibited uredinia of *H. vastatrix* (Silva-Acuña *et al.*, 1999). Thus, each sampling point provided an estimate of number of leaves infected per 50 leaves. This large-scale survey was carried out in the dry season (February) and the wet season (July) of 2007. Previously, it had been established that ants have little preference for any particular species of shade tree, so bias from any species of shade tree is unlikely.

At the local scale, a site was first located with a clear local concentration of *L. lecanii* (as always, associated with a cluster of ant nests in the shade trees) and a 20 × 10-m plot was established in which all individual coffee bushes and all leaves on each bush were counted, recording the incidence of rust-affected leaves. Observing a significant pattern in this first plot, three other sites with clear local concentrations of *L. lecanii* were chosen and line transects extending out from each were established. The distance of each coffee bush from the local concentration was recorded and 100 leaves examined on each bush (beginning from the ninth branch node on the main stem, and moving downward, unless there were fewer than 100 leaves on the bush, in which case all the leaves were counted). The sampling of the bushes was done more or less along the rows of coffee bushes, at a distance of about 0–15 m from the centre of the *L. lecanii* concentration. Sampling was done in areas in which *L. lecanii* was evident but dispersed over many coffee bushes, rather than locally concentrated on one or a few coffee bushes. Using this approach it was reasoned that if *L. lecanii* was contributing to control of *H. vastatrix* through an additional input of spores to the overall system, any pattern of *H. vastatrix* infection associated with *L. lecanii* would be subtle at best, even if the overall controlling effect of *L. lecanii* was a major factor. Sites were also used in which there was an evident local concentration of *L. lecanii* in a matrix of its absence, at least in terms of visual detectability. Also, general areas were sought in which there was a range of incidence of *H. vastatrix*; two areas were found with 4–5% of leaves rusted, and two others with 22–24% of leaves rusted. These four sites were sampled in the wet season of 2007 (July), on a rectangular lattice of 20 × 10 m (site 1), or on transects radiating out from the centre of a *L. lecanii* concentration. Both the radiating transects and the rectangular quadrat incorporated an area that was thought to be free of *L. lecanii* (i.e. physically removed from the focus of *L. lecanii* attack on *C. viridis*). The aim of this methodology was to find any detectable signal that was a function of the distance from the concentration of *L. lecanii*.

Finally, for unknown reasons, *L. lecanii* completely disappeared at one of the four local sites by the wet season of 2008 (there was also a dramatic fall in the local population of *C. viridis* and what appeared to be a debilitated ant colony). Thus, whatever pattern was encountered in 2007 would be expected to disappear if *L. lecanii* was having an

effect on its hosts, so this area was sampled again in July, 2008.

The distance from each coffee bush to the location of the nearest *L. lecanii* concentration was measured. In the case of site 1 there were two local concentrations within the same plot, so the distance to the nearest concentration was measured (in the other three sites there was only a single concentration of the fungus). Standard linear regression analysis was used to establish if there was a significant relationship between the incidence of coffee rust (defined as number of leaves with uredinia) and the distance to the *L. lecanii* concentration. In site 4 the sampling area was extended to about 40 m from the *L. lecanii* concentration to see if the basic spatial pattern held at a larger distance. In the case of the 2008 sample in site 3, the position of the *L. lecanii* source in the previous year was used as the 'centre' and the distance from it to the sampled bush was measured as the independent variable.

Results

Large-scale surveys

Results of the large-scale surveys, along with the position of each ant nest, are shown in Fig. 1. There was an indication that a lower incidence of *H. vastatrix* was associated with higher incidence of ant nests at this very large scale in the dry season of 2007 (note the high density of nests in the upper left of Fig. 1a, along with the relatively low incidence of *H. vastatrix*). However, this was not verified by any statistical test. Casting the problem at several different spatial scales (50 × 50-, 75 × 75- and 100 × 100-m quadrats), there was no statistically significant ($P \leq 0.05$) relationship between ant-nest density and incidence of *H. vastatrix*. However, the relationship became much stronger in the wet season, as shown in Fig. 1b. Since sampling was done on bushes that were either within a restricted area around an *A. instabilis* nest or well-removed from any nest, the data were organized into two categories: 'with' or 'without ants'. The percentage incidence of coffee rust on coffee bushes near to a shade tree with ants versus that on coffee bushes far from any shade tree with ants was statistically significant (*t*-test on arcsin-transformed data, $P = 0.041$, and on a bootstrap resampling test, $P = 0.045$, sample size = 51 bushes near trees with ants, 72 bushes far from trees with ants), which supports the data illustrated in Fig. 1a and b.

Small-scale surveys

In the first small-scale plot there were two local concentrations of *L. lecanii* and a total of 112 sampled coffee bushes. There was a lower incidence of *H. vastatrix* when the sampled bush was nearer to *L. lecanii* (Fig. 2). A linear regression of incidence of *H. vastatrix* versus distance to the closest centre of *L. lecanii* demonstrated a significant relationship ($P < 0.001$) (Table 1).

The three follow-up small-scale surveys, although less detailed with only 100 leaves examined on each bush,

Figure 1 Results of survey of 45-ha coffee plot in (a) dry season and (b) wet season. Size of bubble proportional to incidence of coffee rust, *Hemileia vastatrix*. Solid circles indicate locations of shade trees containing a living nest of the ant, *Azteca instabilis*. Wet season pattern is statistically significant, with higher rust incidence associated with shade trees without ant colonies.

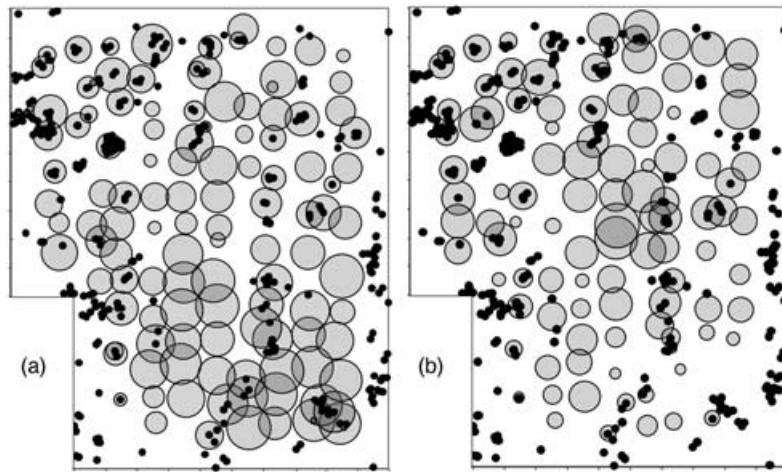


Figure 2 Bubble diagram of site 1 (10 × 20-m plot). Shaded bubbles indicate relative coffee rust incidence (percentage of total leaves with lesions; largest bubble is 27%), on individual coffee bushes; hatched bubbles indicate locations of *Lecanicillium lecanii* infestations on *Coccus viridis*, with relative sizes 1, 2 or 3 representing categories of infestation intensity. Larger circles indicate presumed loci of *L. lecanii* infection origin, to the centre of which the distance measured from each coffee bush.

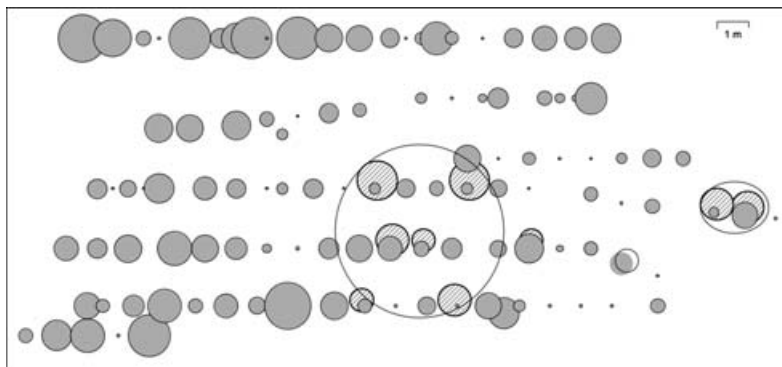


Table 1 Basic statistics for all four small-plot surveys. Site 1 is a 10 × 20-m plot (Fig. 2) and sites 2–4 are smaller plots extending approximately 10 m from the *Lecanicillium lecanii* concentration. Extended 4 is plot 4 extended to 20 m from the *L. lecanii* concentration. Site 3 was surveyed again in 2008 [3 (2008)] after *L. lecanii* had completely disappeared

Site	No. bushes ^a	Rust incidence ^b	Coefficient ^c	R ² ^d	Probability ^e
1	112	4.68	0.66	0.11	< 0.001
2	30	4.36	0.76	0.12	0.01
3	50	22.05	1.63	0.10	0.03
4	25	23.76	2.95	0.35	0.002
Extended 4	51	22.72	0.06	0.03	0.20
3 (2008)	50	4.11	0.001	0.01	0.48

^aTotal number of coffee bushes surveyed in the plot.

^bPercentage of leaves containing at least one rust lesion.

^cRegression coefficient (slope of the least squares best fit of a linear function to the data).

^dCoefficient of determination (broadly speaking, the proportion of the variance explained by the model).

^eProbability of obtaining the regression coefficient by chance.

provided the same qualitative results (Table 1). Standard log scores [(log score – log mean)/log mean] for all bushes at all four sites are shown in Fig. 3. It can be seen that there is a great deal of scatter in the relationship and that the explanatory power of this variable is low.

All four surveys were carried out so that the concentration of *L. lecanii* was within about 10 m of all coffee bushes. An additional group of bushes was surveyed in the case of site 4, involving individual bushes ranging as far as 20 m. When the latter were included, the statistical signi-

ficance of the regression was lost for this site (Table 1), suggesting that the scaling of the input of *L. lecanii* into the environment may be of the order of 10 m. This did not detract, however, from the conclusion that the basic mutualist system of the ant and scale insect contributed to the overall standing crop of *L. lecanii* spores more generally in the plantation.

Site 3 was surveyed in 2008 after *L. lecanii* had disappeared and the relationship with distance to the spot where *L. lecanii* had been the year before was not significant

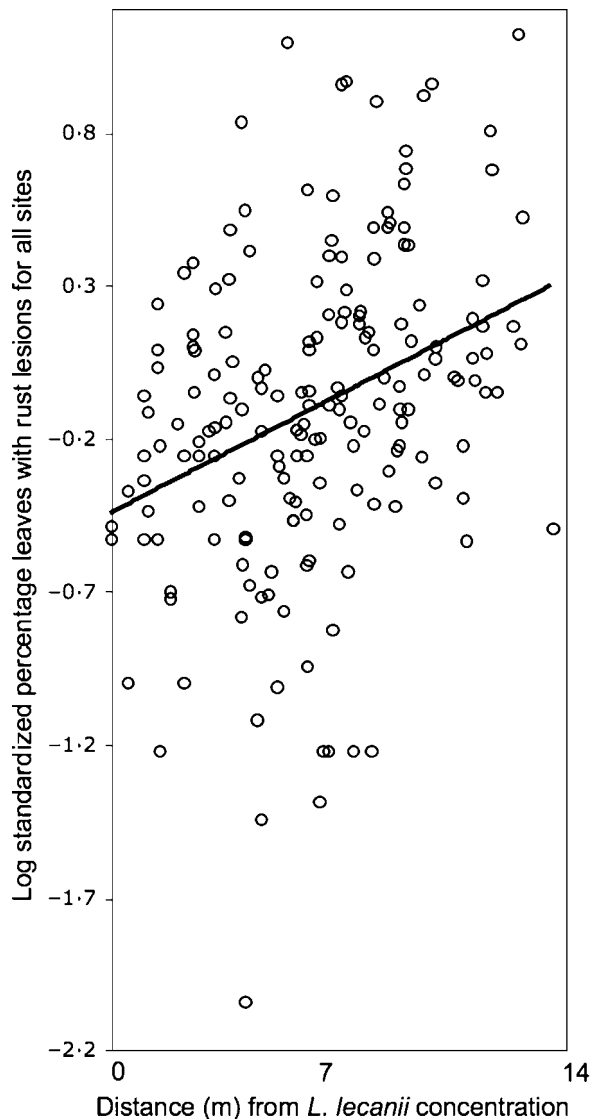


Figure 3 Log-standardized percentage incidence of coffee leaves with rust lesions as a function of distance from *Lecanicillium lecanii* concentrations ($R^2 = 0.108$, $P = 0.00006$), from all mapped points.

(Table 1), suggesting that *L. lecanii* affects the incidence of coffee rust.

Discussion

It appears that there may be at least partial control of *H. vastatrix* by *L. lecanii*, which is enhanced through the existence of the mutualistic association of the ant, *A. instabilis*, with the green coffee scale, *C. viridis*.

Definitive proof that the same isolates of *L. lecanii* that colonize *C. viridis* are able to attack *H. vastatrix* has not yet been reported. Previously, Gonzales *et al.* (1995) reported morphological differences between isolates collected from *C. viridis* and those from *H. vastatrix*. However, Cortez-Madrigal *et al.* (2003) found these

morphological characteristics highly variable among insect-derived isolates. Furthermore, in at least one study (Eskes *et al.*, 1991) the same isolate of *L. lecanii* that attacked *H. vastatrix* was obtained from insects. The results of the present study lend tentative support to the suggestion that the isolates of *L. lecanii* that attack *C. viridis* are also effective at hyperparasitism of *H. vastatrix*. It also supports the view of Andrews (1992) that immigration, as opposed to local population growth, may be important for microbial biological control.

Given the generalized distribution of the ant over this particular coffee farm (and other shaded farms in the region are similar), this mutualism may be a previously unrecognized, yet important, contributor to the generalized dynamics of *H. vastatrix*, adding to the large set of well-known factors already shown to be influential (Avelino *et al.*, 2004). While the specific local effect operates over a short distance (seemingly about 10 m), this effect is useful only to demonstrate that the phenomenon actually exists. From a practical point of view, the mutualism is just one of a host of potential contributors to the overall spore density of *L. lecanii* in the region. Since *L. lecanii* seems to attack a relatively broad range of insects and other fungi (Eskes *et al.*, 1991; Gonzales *et al.*, 1995; Cortez-Madrigal *et al.*, 2003), it is possible that its multiplication occurs on a range of other species, each of which contributes in a small way to the standing crop of *L. lecanii* spores in the environment.

The importance of these results goes beyond the practical issue of biological control. Although it has long been known that natural enemies are important forces in the maintenance of the non-epidemic status of coffee rust, the role of complex ecological interactions has not been emphasized. This study presents evidence for such a role involving a key ant-hemipteran mutualism that operates to allow *L. lecanii* to become locally abundant, thus contributing to the overall standing crop of the spores of this hyperparasite of *H. vastatrix*. These results demonstrate that there is a functional aspect in the associated biodiversity of this agroecosystem, albeit a subtle one.

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