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The epizootiology of a *Metarhizium* infection in mini-nests of the leaf-cutting ant *Atta sexdens rubropilosa*

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Abstract

There is growing interest in the use of entomopathogenic organisms to control leaf-cutting ants (Hymenoptera: Formicidae: Attini). However, the way leaf-cutting ants react as a colony to biohazards is poorly understood. We investigated the effects of *Metarhizium anisopliae* (Metschnikoff) (Deuteromycotina: Hyphomycetes) applied to the foraging arenas of mini-nests (queenless sub-colonies) of the leaf-cutting ant *Atta sexdens rubropilosa* (Forel). Dry spores were applied either alone or mixed with citrus powder, at 0.5 g or 0.05 g per mini-nest. The spores were removed four days after application, and all dead ants removed every three days. Ant numbers near the *Metarhizium* increased as the ants attempted to clean up the biohazard. The ants attempted to place the spores in piles, which they then covered over with other material. They were able to deal with the low doses in this way, but the high doses overwhelmed them. All treated mini-nests suffered increased ant mortality during the first ten days after application. This mortality was particularly high in the media worker caste which had played the major role in attempting to clean up the spores. Foraging activity decreased, as did the health of the fungus gardens. The mini-nests exposed to the low dose of spores mixed with citrus powder then recovered fully. The health of the other treated mini-nests declined gradually until around 26 days after application, when they began deteriorating sharply. However, the decline of these mini-nests after day 26 was not due directly to the pathogenic action of the *Metarhizium*, nor to the initial ant mortality it had caused. The results suggest that the social stress caused by even such a short-lived *Metarhizium* epizootic was sufficient to cause the decline and ultimate death of the mini-nests. This has important implications for the control of leaf-cutting ants. It also demonstrates how important the social homeostasis of the colony is to leaf-cutting ants.

Introduction

Leaf-cutting ants of the genera *Atta* and *Acromyrmex*, are dominant herbivores of the neotropics (Hölldobler & Wilson, 1990). The material they harvest is used to cultivate an obligate ectomutualistic fungus within the nest. This fungus produces specialised structures called gongylidae that are an essential protein-rich food source for the ant larvae (Martin et al., 1969; Quinlan & Cherrett, 1979; Bass & Cherrett, 1995). The ants can account for as much as 50% of the total herbivory in some areas (Blanton & Ewel, 1985), and are one of the most destructive pests in many of the regions where they occur (Weber, 1972; Cherrett, 1986a).

Most modern control methods are based on the application of large quantities of insecticides, which are costly and hazardous to the environment (Cherrett, 1986a). Much research has therefore been carried out with the aim of finding alternative methods of control. One possibility that has emerged recently is the use of biological control agents.

The effectiveness of entomopathogenic microorganisms at controlling a wide variety of insects is now

well established. The fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Metschnikoff) (Deuteromycotina: Hyphomycetes) are among the agents that have been the most widely used (Evans, 1989; Zimmerman, 1993; Hajek & St. Leger, 1994). However, relatively little work has been carried out on the microbial control of ants in general, and of leaf-cutting ants in particular. This is in spite of the fact that entomopathogens can commonly be found infecting ants (Evans, 1974; Samson et al., 1988; Evans, 1989). *Beauveria bassiana* has been isolated from workers of *Atta sexdens piriventris* (Diehl-Fleig et al., 1992b) and from queens of *A. sexdens rubropilosa* (Alves & Sosa Gómez, 1983). In the latter study, *Metarhizium anisopliae* was also found to be infecting the queens of *A. sexdens rubropilosa*, and this fungus has since been isolated from queens of *A. bisphaerica* (C. W. Jackson & W. O. H. Hughes, unpubl.).

In laboratory studies it has been conclusively demonstrated that *Metarhizium* sp. and *Beauveria* sp. can cause 100% mortality of isolated groups of leaf-cutting ants (Kermarrec & Decharme, 1982; Kermarrec et al., 1986; Lima et al., 1986; Alves & Sosa Gómez, 1983; Silva & Diehl-Fleig, 1988; Blowers, 1994; Jaccoud, 1996). However, initial experiments found the fungi to be far less effective when applied to whole colonies (Kermarrec & Decharme, 1982; Kermarrec et al., 1986). This lack of efficacy against whole nests has been attributed to several behavioural and chemical factors.

Leaf-cutting ants are extremely effective at recognising dangerous material such as entomopathogenic organisms (Kermarrec & Decharme, 1982; Kermarrec et al., 1986). They will attempt to remove any contaminated material from the nest, including dead or dying nest-mates, in order to prevent spread of infection to other members of the colony (Kermarrec et al., 1986). Ants will engage in intensive self-grooming to rid themselves of any alien fungal spores (Kermarrec & Decharme, 1982; Kermarrec et al., 1986; Diehl-Fleig, 1991). The spores are agglutinated into small pellets in the infra-buccal pocket (Kermarrec et al., 1986). Here they are covered in chitinolytic secretions from the labial glands, which inhibit their germination (Febvay et al., 1984). In addition, both the ants and their mutualistic fungus are reported to produce various antibiotic compounds which help prevent alien organisms from surviving within the nest environment (Kermarrec & Decharme, 1982; Kermarrec et al., 1986; Diehl-Fleig, 1991; Knapp et al., 1994; Knapp, 1995).

In spite of these defensive mechanisms, successful biological control of leaf-cutting ant nests has been achieved. Silva & Diehl-Fleig, (1988) found that the foraging activity of adult *Atta sexdens piriventris* nests was greatly reduced sixty days after spore suspensions or powder formulations of *Beauveria* or *Metarhizium* were applied to nest entrance holes. Work on field colonies of *Acromyrmex* achieved up to 87% control with *Beauveria* spores, formulated in rice granules, applied directly on to the fungus garden itself (Diehl-Fleig et al., 1993; Silva et al., 1993). 100% control of *A. sexdens rubropilosa* nests has been reported using suspension and powder formulations (Lima et al., 1986). However, such inundative applications directly to the nest are time consuming, and would be impractical as a viable control strategy for large areas. A more efficient alternative is to formulate the fungal spores as a bait for the ants to transport to the nest themselves. Although ants generally recognise entomopathogens, their presence can be disguised by the addition of attractant fruit extracts, resulting in the bait being transported by the ants (Blowers et al., 1992; Specht et al., 1994). *Beauveria bassiana*, when formulated in baits with orange peel, controlled 72% of the 50 *Acromyrmex* colonies in an area of *Eucalyptus* plantation (Diehl-Fleig et al., 1992a).

Entomopathogenic fungi, therefore, clearly have a great deal of potential as control agents of leaf-cutting ants. However, the majority of work to date has concentrated almost solely on achieving control and there has been no detailed examination of the epizootiology of an entomopathogen infection within colonies of leaf-cutting ants. In this study, we used queenless sub-colonies of a leaf-cutting ant nest, termed 'mini-nests' (Loeck et al., 1991, 1993, 1994), to simulate the effects of an entomopathogen infection upon leaf-cutting ant colonies.

Materials and methods

The method of culturing small, queenless colonies of leaf-cutting ants was first developed by Loeck et al. (1991, 1993, 1994). Such 'mini-nests' can survive for up to four months, or indefinitely if new workers are periodically added from the parent colony to replace those that had died in the mini-nest. Furthermore, these mini-nests appear to show many of the characteristic types of behaviour of a normal, queen-right colony (Loeck et al., 1991, 1993, 1994).

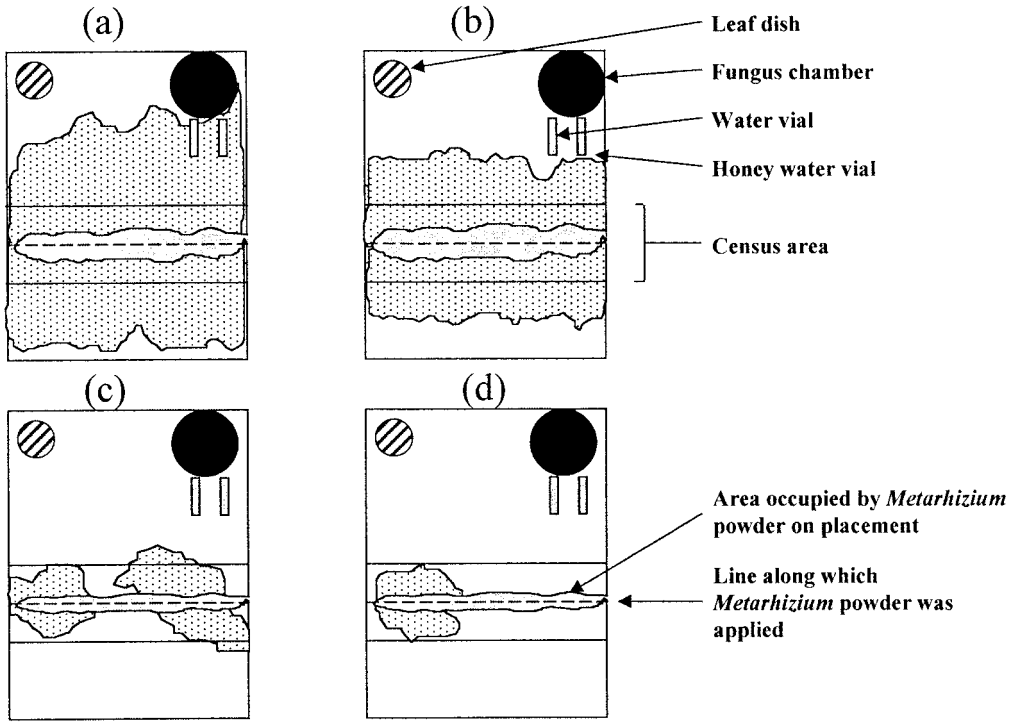

Figure 1. Representative diagrams of the layout of the *A. sexdens rubropilosa* mini-nests, showing spread of *Metarhizium* powder (hashed area) in the treated mini-nests after 24 h (a = 0.5 g dry spores, b = 0.5 g dry spores + 0.5g citrus powder, c = 0.05 g dry spores, d = 0.05 g dry spores + 0.05 g citrus powder).

During this work an adapted technique was used, in which the entire set-up of each mini-nest was contained within a single clear plastic box, of 28 × 16 × 9 cm (Figure 1). The boxes were lidded in order to reduce disturbance to the mini-nests. A 75 ml sample of fresh fungus garden, together with any associated ants or brood, was taken from a parent colony of *Atta sexdens rubropilosa* and placed in a clear plastic cup of 6 cm height and approximately 300 ml volume. This was then inverted on to a petri dish containing a layer of cotton wool, a filter paper disc, and a disc of 0.5 mm nylon mesh. Two 1 cm diameter exit holes were cut in the side of the cup, and the chamber placed at one end of the box. A 10 ml vial was connected to the chamber with transparent PVC tubing in order to serve as a dump chamber, and two 4.5 ml vials placed inside the box. These smaller vials contained either distilled water or a honey water solution (10% honey by volume), and had cotton wool wicks placed in them. Nineteen mini-nests were set up in this manner, with all having been collected from the same nest of *A. sexdens rubropilosa* during a 30-min period. This parent colony had been collected from Viçosa, Minas Gerais, Brazil and maintained in the laboratory for six years under conditions of approximately 25 °C, 60% r.h., and L12:D12 cycle.

During the experiment, the mini-nests were kept at 25 °C, 90 ± 2% r.h., and L12:D12 cycle. Rose (*Rosa* spp.) and bramble (*Rubus fruticosus*) leaves were offered daily, and any non-foraged material was collected and recorded. The water and honey water vials were replaced every seven days.

In this study, strain ARSEF 551 of *Metarhizium anisopliae* was used to examine the effect of an entomopathogenic fungus on mini-nests of *A. sexdens rubropilosa*. This strain was originally isolated from spittlebug (Homoptera:Cercopidae) from Brazil (Humber & Soper, 1986), and had previously been found to be highly pathogenic to isolated groups of leaf-cutting ants (Jaccoud, 1996). The fungus was tested in two formulations, either dry spores alone (DS), or dry spores mixed with citrus powder at a ratio of 1:1 (w:w) (CP). This mixing was not found to inhibit the germination of the *Metarhizium* (Jaccoud, 1996). Each formulation was applied at two doses of three week old *Metarhizium* spores, a high dose of 0.5 g per mini-nest or a low dose of 0.05 g per mini-nest. In the CP treatments therefore, the total quantity

of powder applied was either 1 g or 0.1 g, although the weight of spores was the same as in the DS treatments. In all cases the powder was placed in a horizontal strip, along a line 10.5 cm from one end of the box, with the colony fungus chamber being located at the opposite end (Figure 1). Four days later the powder was, as far as possible, removed from the mini-nests. Four mini-nests were randomly allocated to each of the four treatments, and a further three were used as controls and had no powder added to them.

During the experimental period a number of records were made. An hour prior to the experiment, and at 1,3, 6, 9, 12, 24 and 48 h after application, the number of ants within a census area were recorded. This number was the mean of six counts carried out for each mini-nest at each time. The census area was the region 3.5 cm to either side of the line along which the fungus powder had been applied (Figure 1). Ants counted were divided into three castes depending on their estimated head widths. Minor workers were 1.2 mm or less, medias between 1.2 and 1.6 mm, and maximas were greater than 1.6 mm.

The spread of the *Metarhizium* powder over the mini-nest floor (termed the 'arena') was assessed in the treated mini-nests at 9,24, 48 and 72 h after application. This was done with the aid of a grid drawn on the bottom surface of the mini-nest. In order to establish if spores had reached the fungus garden itself, small samples of the symbiotic fungus were taken 10 days after application, and placed on selective media plates [3.5% agar, 1% chloramphenicol, 0.5% cyclohexamide, and 10 mg/ml dodine (Liu et al., 1993)]. Any fungal growth observed after 12 days was collected and placed on media plates (5 g l^{-1} mycological peptane, 2 g l^{-1} yeast extract, 2 g l^{-1} malt extract, 8 g l^{-1} sucrose and 12 g l^{-1} agar) for growth and sporulation. Twelve days later, spores were collected and applied to 10 ants which were placed in humidity chambers for pathogen recovery.

In addition, three measurements were made of the health of the mini-nests. Dead ants were removed and the mortality recorded on days -1, 1, and every three days thereafter, with the dead ants from every alternate sampling being placed in humidity chambers and assessed for pathogen sporulation. Foraging activity was measured daily from day -2, as the percentage of offered leaf material that was harvested by the ants within 24 h. Finally, the health of the fungus garden was assessed every four days, beginning on day 2. The fungus gardens developed in the same way as those in normal colonies, with old, brown fungus at the bottom and fresh, grey material being added to the top (Weber, 1972; Bass & Cherrett, 1996). This allowed the gardens to be divided into regions according to its state of development, represented by its colour. Each region was ranked by this means (Sokal & Rohlf, 1995), going from dark grey, freshly implanted fungus (rank 4), through white and yellow mature fungus garden (ranks 3 and 2), to old brown fungus (rank 1) and dead fungus (rank 0). The area of each region was estimated using a grid drawn on the side of the mini-nest, and another grid on the lid of the mini-nest was used to estimate the total area of the gardens from overhead view. To get an overall estimate of the health of each fungus garden we used the formula:

$$h = (\sum da)o,$$

where h is the overall health of the garden, d is the development rank of each region, a is the area of that region, and o is the area of the garden from overhead view. This gave a score for the health of the fungus gardens based primarily upon their estimated volume. Gardens with a large volume of fungus would score higher, and be described as healthier than gardens with a low volume of fungus. It also takes into account the gardens state of development. Clearly a garden made up only of dead fungus could not be described as healthy. Using this equation, a garden with even a large volume of old or dead fungus will score lower than one made up of significant amounts of fresh and mature fungus.

Statistical analysis

For the most part, the data was analysed using one-way repeated measures analysis of variance (ANOVA). By examining the interaction between treatment and time, this tested the null hypothesis that the mini-nests all changed in the same way over the measured period regardless of the treatment they were exposed to. Repeated measures designs are appropriate where measurements have been taken of the same replicates at several time points (Green, 1993; Paine, 1996). The ant mortality data was analysed with a one-way ANOVA to examine if the treatments varied in mortality at the end of the 37 day experimental period. The count data was log-transformed prior to analysis, in order that it more closely met the assumptions of a normal distribution and homogeneity of variance. Where the data was proportions, as in the mortality and foraging activity records, it was arcsine-transformed prior to analysis. The caste ratios of the ant populations were compared using G tests.

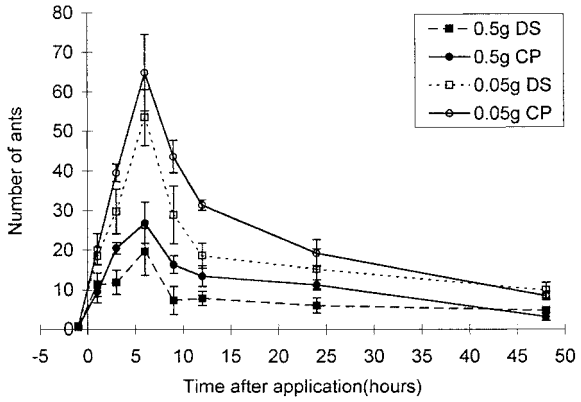

Figure 2. Mean (± s.e.) ant activity recorded in the census areas of treated *A. sexdens rubropilosa* mini-nests during the first 48 h after application of the *Metarhizium* powder (DS = dry spores, CP = dry spores + citrus powder).

Results

Ant reaction and cleaning behaviour. In all four of the treatments, there was an immediate increase after the addition of the *Metarhizium* spores, in the number of ants observed in the census areas (Figure 2). Prior to application of the powder there were very few ants in the census areas. In the controls, activity remained at this low level throughout the experiment and they were therefore excluded from the analysis. In the treatments, the numbers observed increased to a maximum at 6 h after application and then decreased. There were still at least 5 times as many ants present at 48 h as there had been before treatment. The ant response over time differed significantly between the treatments ($F_{21,84} = 3.51$, $P<0.001$). The CP treatments were consistently associated with slightly more ants than the DS treatments, and the low dose treatments were associated with considerably more ants than the high doses.

Ants within the census areas were frequently seen moving around rapidly and engaging in intensive self-grooming, as well as exploring the powder with their antennae and mouthparts. In addition, physical recruitment was seen on a number of occasions. This involved one ant, normally a maxima, picking up another individual and carrying it to the fungus powder. The transported individual was then deposited here, and would either leave or engage in the same behaviours as the other ants near the *Metarhizium* spores.

Prior to the *Metarhizium* powder being applied, most of the ants observed within the census areas were media workers. The average caste make-up at this time was 22 ± 6.4% minors, 62.5 ± 8.3% medias, and 15.0 ± 4.9% maximas. This was significantly different from the overall populations which were on average 73.4 ± 1.8% minor workers, 19.4 ± 1.4% medias and 7.2 ± 0.7% maximas ($G = 14.6$, $df = 2$, $P<0.001$). In all treated mini-nests, following application of the *Metarhizium* powder the percentage of ants in the census areas that were minors increased to an average of 41.3 ± 2.1%, while the percentage that were medias and maximas decreased to 50.5 ± 2.5% and 8.2 ± 2.7%, respectively. However, this change was not statistically significant ($G = 3.64$, $df = 2$, $P>0.05$), and the proportions after application were still significantly different from the overall populations ($G = 115.42$, $df = 2$, $P<0.001$). Media workers were therefore the most abundant caste involved in cleaning up the *Metarhizium*. They also made up a far greater proportion of the ants that responded to the powder being applied than would have been expected, given that 73% of the overall ant populations were minors.

The ants attempted to deal with the *Metarhizium* spores by concentrating the spores into piles. They also covered over the spores with a variety of different materials, including pieces of leaf, filter paper, and cotton wool. The change over time in the spread of powder differed significantly between treatments ($F_{9,36} = 26.6$, $P<0.001$). Within 9 h of the *Metarhizium* being applied, the ants in the 0.05 CP treatment had reduced the powder to piles occupying, in total, only 9 ± 2% of the arena area (Figure 3). By 24 h after application the powder in the 0.05 DS treatment had also been reduced, to 19 ± 4% of the arena (Figures 1 and 3). However, in both the high dose treatments the area covered by the powder had increased considerably, and continued to do so until it occupied 84 ± 3% in the 0.5 DS and 66 ± 3% in the 0.5 CP after 72 h.

Mini-nest health

Ant mortality. In all *Metarhizium* treatments there was an initial sharp rise in mortality, peaking at day 7 (Figure 4). The cumulative mortality increased only slowly thereafter until day 28 when it began to rise sharply in the DS and 0.5 CP treatments. By day 34 there was 100% mortality in the 0.5 DS mini-nests, while the 0.5 CP and 0.05 DS treatments averaged 64.5±15.4% and 45.2±17.3% respectively by the end of the experimental period. There was very little mortality in the 0.05 CP mini-nests after the initial peak which had ended by day 16. The control mortality was

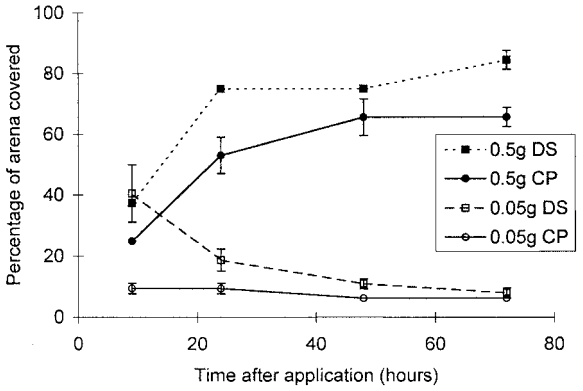

Figure 3. Mean (± s.e.) spread of *Metarhizium* powder in the treated *A. sexdens rubropilosa* mini-nests during the first 72 h after application of the powder, expressed as percentages of the arena areas covered (DS = dry spores, CP = dry spores + citrus powder).

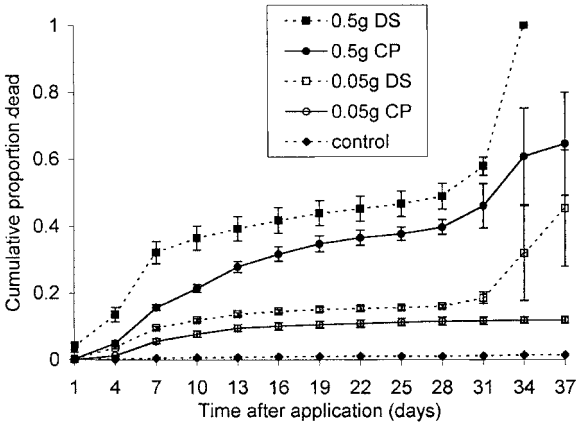

Figure 4. Mean (± s.e.) cumulative mortality of *A. sexdens rubropilosa* mini-nest populations, expressed as proportions of total ant populations (DS = dry spores, CP = dry spores + citrus powder).

extremely low throughout the experiment, with only 1.4 ± 0.1% of the ant population having died after 37 days. The effect of treatment on the final mortality was highly significant ($F_{4,14} = 87.7$, $P<0.001$).

Pathogen recovery from the dead ants was consistently high during the first four weeks, with *Metarhizium* sporulating from greater than 80% of cadavers for all treatments. However, from day 31 the percentage recovery decreased substantially. On day 37 it was 30% in the 0.5 CP treatment and less than 10% in the low doses, with there being considerable within-treatment variability. Saprophytic fungi were increasingly observed on the ant cadavers towards the end of the experimental period. However, this did not appear to be the reason for the reduced *Metarhizium* sporulation.

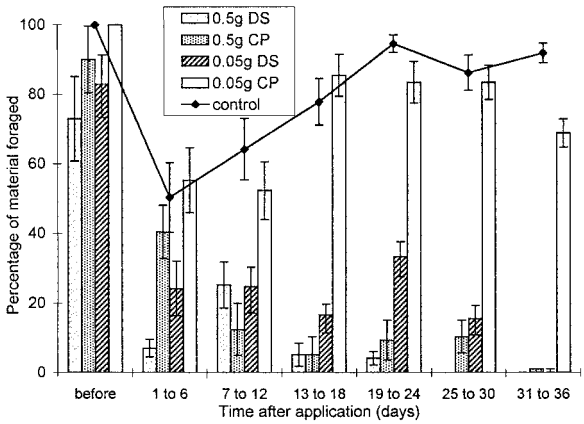

Figure 5. Mean (± s.e.) foraging activity of *A. sexdens rubropilosa* mini-nests. Each bar or point represents the mean percentage of leaf material harvested per day for a six day period. Leaf material was replaced on a daily basis. The control data are shown as a line for clarity (DS = dry spores, CP = dry spores + citrus powder).

On day 1, at least 50% of the dead ants in all treatments were minor workers with most of the remainder being medias. By day 7 this had changed significantly to only approximately 20% being minors while up to 70% were media workers ($G = 15.3$, $df = 2$, $P<0.001$). In the DS and 0.5 CP treatments the percentage of dead ants that were minor workers then gradually increased to about 90% by the end of the experiment.

Foraging activity. There were significant differences between the treatments in the way their foraging activity changed over the course of the experiment ($F_{144,504} = 2.27$, $P<0.001$). In all mini-nests, including the controls, the amount of material foraged declined initially up to day 6 (Figure 5). The control and 0.05 CP mini-nests then harvested increasingly more material, and consistently foraged greater than 65% of the leaf material per day after day 13. In the 0.5 DS treatment, the amount of material foraged only recovered to 25.2±6.6% before decreasing once again with none of the material being harvested at all on all but five days after day 10. The foraging activity of the mini-nests exposed to the 0.5 CP and 0.05 DS treatments did appear to recover to somewhat higher levels of as much as 10.4 ± 3.7% and 33.3 ± 5.8%, respectively. However, it was observed that much of this material was left at the base of the nest chambers and was either incorporated very slowly into the fungus garden, or not at all. This was termed ‘false foraging’, and it occurred to some degree in all mini-nests. Although it was not quantified, there was clearly

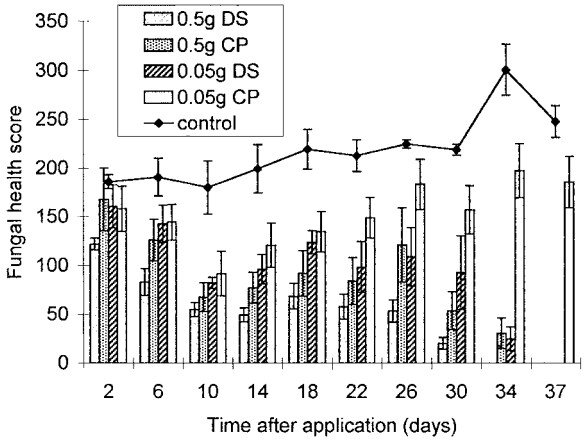

Figure 6. Mean (± s.e.) health scores of *A. sexdens rubropilosa* mini-nest fungus gardens. The control data are shown as a line for clarity. (DS = dry spores, CP = dry spores + citrus powder).

a greater quantity of material left unincorporated in the DS and 0.5 CP mini-nests, than in those that received the 0.05 CP treatment or were controls. As the mini-nests in the former three treatments were also transporting considerably less material in the first place, the importance of false foraging in their case would be especially significant.

Fungus garden health. There were no significant differences between the treatments in the health scores of their fungus gardens prior to the experiment ($F_{4,14} = 1.10$, $P>0.05$). However, the treatments did differ significantly in the way their fungus gardens changed in health after application of the *Metarhizium* ($F_{36,126} = 8.66$, $P<0.001$). During the 37 day experimental period, the health of the control fungus gardens increased virtually throughout (Figure 6). However, in all of the mini-nests that had been exposed to one of the *Metarhizium* treatments, the fungus gardens health scores decreased up to day 10. After this initial decline the 0.05 CP mini-nests recovered and their health scores increased thereafter. Although some recovery also occurred in the DS and 0.5 CP treatments, it was to a much lesser degree. From day 26, in the high dose treatments, and day 30 in the 0.05 DS treatment, the health of the fungus gardens started declining once again. They were dead by day 34 in the 0.5 DS treatment, and by day 37 in the 0.5 CP and 0.05 DS treatments.

Discussion

The placement of the *Metarhizium* spores resulted in a rapid response from the ants. Whereas in other work leaf-cutting ants have been found to be repelled by the presence of an entomopathogenic agent (Machado et al., 1988; Diehl-Fleig & Lucchese, 1991), in this case ant numbers near the spores increased. It seems likely that this was a result of the small, enclosed area that made up the mini-nests. Under natural conditions ants will often switch their activity to a different entrance hole in order to avoid the hazard (Silva & Diehl-Fleig, 1988; Diehl-Fleig, 1991; Diehl-Fleig & Lucchese, 1991). In extreme cases, colonies may even migrate to new locations (Machado et al., 1988). Neither of these types of behaviour would be possible in the enclosed environment of the mini-nests used here. The ants may therefore have been forced to adopt an alternative strategy of attempting to clear up the biohazard instead of avoiding it.

One interesting feature of the increase in ant numbers near the powder, was the way ants picked up and carried other individuals to the fungus powder. Physical transport of one individual by another has been observed in many ant species (Hölldobler & Wilson, 1990) and is generally associated with the migration of nests (Möglich & Hölldobler, 1974). The only previous report of it in leaf-cutting ants was by Gamboa (1975) who commonly observed ants being transported out of nests of *Acromyrmex versicolor*. However, the reason for this was unclear. In the present study, two possible purposes suggest themselves. Firstly, the transported ants may have been individuals contaminated by the *Metarhizium*, which were therefore being removed from the fungus chamber to prevent them infecting other individuals. Alternatively the ant carrying behaviour may have been a way of actively recruiting individuals to where they were required. Upon being deposited in the area of the *Metarhizium*, many of the transported ants did in fact then exhibit cleaning behaviour. The number of individuals involved in dealing with the powder was thus increased by this unusual behaviour.

The number of ants counted in the census areas was consistently higher in the citrus powder treatments than in the dry spore treatments. This was probably due to the arrestant effect that citrus is known to have on many species of leaf-cutting ant, including *A. sexdens* (Cherrett & Seaforth, 1970; Mudd et al., 1978; Cherrett, 1986b). There was also a dose-dependent relationship, with more ants being counted in the low

dose treatments. Although ants were occasionally observed walking over the powder itself, it seems likely that they would prefer to clear it up from the edges (Jackson, personal observation). This means that the actual area within the census area available for the ants to stand or walk would be less in the high dose treatments than in the low doses, because the high doses covered a larger area on placement (Figure 1). The census areas of the high dose treatments would therefore be expected to contain fewer ants than those of the low doses.

The ants were able to deal effectively with low doses of *Metarhizium*, and had the spores concentrated into one or two piles within 24 h. This allowed them to cover over the spores and prevent further ants becoming exposed to them. The larger dose of 0.5 g appeared to overwhelm the ants, and was actually spread further by them during their attempt to clear it up. In both low and high doses, the ants dealt with the citrus powder formulation more successfully than with dry spores alone. The citrus powder particles were considerably larger than the *Metarhizium* spores and so would have been easier for the ants to manipulate. Given the extremely high humidity in the mini-nests, it is also probable that the spores would have clumped together to some degree with the citrus powder particles. This would have further simplified the cleaning procedure for the ants.

In all the treated nests, the first ten days after application were characterised by a high ant mortality, reduced foraging activity, and a decline in the health of the fungus garden. After day ten, the health of most mini-nests stabilised, while that of those exposed to the 0.05 CP treatment recovered. Then from day 26, the health of the DS and 0.5 CP mini-nests declined rapidly to the point of death of the fungus gardens in all cases, and of the ant populations in many of them. The control mini-nests suffered very little ant mortality and remained healthy throughout the experiment, with high foraging activity and fungal health.

It seems likely that the high ant mortality during the first ten days was related to the high level of cleaning behaviour shown by the ants during the first 24 h in particular, as demonstrated by the number of ants counted within the census areas. Further evidence that this was the case comes from the caste make-up of the dead ants. Most of the ants that died during this initial period were media workers, and this was also the caste that was observed in the census area to the greatest degree. It is thought that the media workers form a generalist caste that carry out a variety of roles in the colony, including degradation of vegetation and disposal of refuge (Wilson, 1980). The fact that media workers made up the majority of the ants that responded to the application of the *Metarhizium*, and more importantly, that this number was far greater than their proportion in the overall populations, suggests that they played the major role in cleaning up the spores. They would therefore have been the group most exposed to the biohazard, and consequently the caste that would be expected to suffer the highest mortality.

There was a sharp drop in foraging activity during the first ten days. While this also occurred in the control mini-nests, it happened to a much greater degree in those that had been exposed to the *Metarhizium*. It seems extremely likely that the subsequent drop in the health of the fungus garden was a result of this decrease in foraging activity. It could also have been due to the death, or diversion to other duties, of ants that would normally have been caring for the mutualistic fungus.

From day 26 in the 0.5 DS and 0.5 CP treatments, and day 30 in the 0.05 DS treatment, the health of the fungus gardens started to decline. This was followed very rapidly by a dramatic increase in ant mortality that resulted in the death of all the 0.5 DS mini-nests by day 34. By the end of the experimental period on day 37, two of the 0.5 CP mini-nests had also died, with both of the others having suffered greater than 45% mortality. By this time, the fungus gardens of these two surviving 0.5 CP mini-nests had died, as had those of the 0.05 DS mini-nests. This, combined with the increasing ant mortality that these mini-nests exhibited, strongly suggests that they would have died within about another week. At no stage after day 10 was there any sign of a decline in health of the 0.05 CP mini-nests, and it would seem that the ants had been able to deal with this treatment effectively.

It is interesting to speculate as to the probable reasons for the decline and death of the mini-nests of the DS and 0.5 CP treatments during the last 11 day period. Unlike during the earlier part of the experiment, pathogen recovery from ants that died during the last 11 days was relatively low. In fact, there is no reason for the pathogenic action of the *Metarhizium* to have caused such a substantial increase in mortality at this time. The *Metarhizium* spores were largely removed four days after application, and as all ant cadavers were also removed every three days, there was no way for the *Metarhizium* to have built up in the mini-nests.

There must therefore have been another cause for this increase in mortality.

The health of the fungus gardens did begin to decline immediately before the ant mortality increased, suggesting the ants may have starved as their gardens died. However, adult ants get only 9% of their energy requirements from the mutualistic fungus, with the remainder being obtained from leaf sap (Quinlan & Cherrett, 1979; Bass & Cherrett, 1995). They can survive for around 20 days solely on the water and sugar sources that were permanently present in the mini-nests (Bass & Cherrett, 1995; Jaccoud, 1996). If the observed decrease in foraging on leaves, though, was accompanied by a decrease in foraging on the sugar and water sources, which seems plausible, then starvation remains a likely cause of the ant mortality during the last 11 days.

Although the set-up used in this experiment was highly artificial, its results demonstrate a number of features of the social biology of leaf-cutting ants that are likely to be true of natural colonies as well. It has often been emphasised that their social biology makes leaf-cutting ants extremely efficient at eliminating biohazards (Febvay et al., 1984; Kermarrec et al., 1986; Diehl-Fleig, 1991; Knapp et al., 1994). Indeed in this study the ants were seen to react extremely rapidly to the presence of *Metarhizium* close to their fungus chamber, and in the case of the low dose treatments, they were able to isolate and cover over the fungal powder within 24 h.

However, it would appear that their complex social biology also works against them to some degree. Firstly, *Metarhizium* spores were isolated from the fungus gardens, ten days after application. So, in the process of cleaning up the spores, the ants had themselves infected the main nest chamber. Furthermore, these live *Metarhizium* spores had survived within the fungus chambers in spite of the barrage of anti-microbial compounds that leaf-cutting ants and their mutualistic fungus are known to produce (Kermarrec & Decharme, 1982; Kermarrec et al., 1986; Diehl-Fleig, 1991; Knapp et al., 1994; Knapp, 1995).

Secondly, the mini-nest mortality was not due to the *Metarhizium* infecting and killing every individual in the population. Rather, it was the result of some process set in motion by the early *Metarhizium* epizootic. The decline of the mini-nests was not explained by the high initial ant mortality either. The mini-nests in the 0.05 CP treatment suffered an initial level of mortality that was almost the same as that seen in the 0.05 DS mini-nests. Yet the latter mini-nests eventually declined drastically in health while the former recovered fully.

One of the main characteristics of social insect colonies is their social homeostasis (Hölldobler & Wilson, 1990). The *Metarhizium* epizootic would have placed a number of stresses on this. Ants would have been diverted from other duties to cleaning up the *Metarhizium*. Foraging was observed to be disrupted, and trophallaxis and nest hygiene behaviour probably were as well. The fact that it took the ants longer to clean up the 0.05 DS treatment than the 0.05 CP treatment, suggests that they found it harder to deal with, and that it therefore placed more stress on the mini-nests. It would appear that this higher level of stress was sufficient to break down the social homeostasis of the mini-nests and kill them. From a control perspective this is particularly interesting. It suggests it is not necessary to kill every ant directly with the control agent. Causing a sufficient level of social disruption to the colony may kill it just as effectively. This is one aspect that clearly deserves further investigation.

The majority of studies to date have concentrated on either the overall success or failure of attempts to control leaf-cutting ant colonies with entomopathogens, or on the specific defences that the ants have evolved. This study has gone some way towards providing a link between these two levels. Mini-nests are highly artificial, but are nevertheless a useful tool in studying leaf-cutting ants. Further work both with mini-nests, and ideally at the more representative level of whole colonies, is required before we will have a true understanding of what is happening within leaf-cutting ant colonies that are exposed to biohazards.

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