Fungal enzymes transferred by leaf-cutting ants in their fungus gardens

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Leaf-cutting ants finely fragment the leaf material that they bring to the nest and place faecal droplets on this substrate before incorporating it in the upper part of the fungus-garden. The faecal droplets contain enzymes of which some have been shown to be of fungal origin. Here we explicitly address the enzymatic activity of faecal droplets in the leaf-cutting ants *Acromyrmex echinatior* and *Atta colombica*. We used isoelectric focusing and specific staining to show that faecal droplets of both species contain carboxymethylcellulases, laccases, proteases, and pectinases (both pectin esterases and pectin lysases) and we demonstrate that these enzymes originate from the symbiotic fungus and not from the ants themselves. The level of activity of fungal pectin lyase in faecal droplets indicates that fungal enzymes may be protected and possibly concentrated during their passage through the ant gut. This would imply that enzymes that are transferred by ants from mature parts of the fungus gardens may play an important role for the colonization of new substrate by new mycelium of the same fungal clone.

INTRODUCTION

All ants in the tribe Attini are obligately dependent on the cultivation of symbiotic fungi for food. The most derived genera, *Atta* and *Acromyrmex*, are known as the leaf-cutting ants and are dominant herbivores and agricultural pest in tropical and subtropical America (Cherrett, Powell & Stradling 1989). Their symbiotic fungus *Leucoagaricus gongylophorus* (*Basidiomycota*) has been described from rare fructifications on fungus-gardens (Fisher, Stradling & Pegler 1994, Pagnocca *et al.* 2001, Mueller 2002). The leaf-cutting ant workers collect fresh leaf fragments and transport them to their underground fungus-gardens where the fragments are chewed into a pulp. During this process the ants ingest plant sap (Littledyke & Cherrett 1976) and place faecal droplets on the plant material before inoculating it with the symbiotic fungus and incorporating it in the fungus-garden (Weber 1972).

In the mature parts of the garden the fungus produces staphylae, bundles of specialized swollen hyphal tips known as gongylidia. The gongylidia are fed to the larvae and the workers also chew on them and ingest the cytoplasm (Quinlan & Cherrett 1979). The fungus thus plays an essential nutritional role for the ants, as the larvae are raised solely on this fungal diet and the workers depend partly on it to supplement their diet of plant sap (Quinlan & Cherrett 1979, Bass & Cherrett 1995).

In the course of evolution, the ingestion of fungal material may have acquired novel functions in the symbiosis in addition to direct nutrition, for example by the acquisition of enzymes. This subject has been addressed in earlier studies of leaf-cutting ants. Faecal droplets of *Atta colombica* have been found to degrade chitin, CMC (carboxymethylcellulose), pectin, starch and xylan (Martin, Gieselmann & Martin 1973, Martin *et al.* 1975) and proteases in the faecal droplets of *Atta texana* were shown to be identical to proteases in cultured mycelium of the symbiotic fungus (Boyd & Martin 1975a, b). The presence of fungal proteases in faecal droplets suggests that part of the ants' preparation of the leaves for colonization by the fungus is to distribute the fungal enzymes on the new substrate (Boyd & Martin 1975b, Mueller 2002).

The aim of the present study was to quantify the plant degrading enzyme activity in faecal droplets of *Acromyrmex* and *Atta* leaf-cutting ants to clarify

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whether the fungal enzymes in faecal droplets are an insignificant side effect of the ingestion of fungal material, or whether the enzymes are protected against degradation in the ant gut.

MATERIALS AND METHODS

Ants and fungal material

Colonies of *Atta colombica* and of *Acromyrmex echinatior* were collected in Gamboa, Panama in 1996 and 1998 and maintained in the laboratory under standard conditions of 25 °C and 70% RH (Bot & Boomsma 1996). To obtain pure cultures of the symbiotic fungus, mycelium from the upper part of fungus gardens of a single colony of each species was placed on potato dextrose agar plates (Difco) and incubated in darkness at 25 °. White mycelia growing from the garden material were transferred to new dishes, where the symbiotic fungus could be identified by their simple hyphae without spores or conidia and by the development of gongylidia after one or two weeks (Pagnocca *et al.* 2001). The cultures have been placed in mineral oil for storage as AcoP1 and Ae56 at the Botanical Institute, University of Copenhagen (Cazin, Wiemer & Howard 1989).

Proteins were extracted from mycelium of the symbiotic fungi on PDA by grinding 50 mg mycelium of the pure cultures in 1.5 ml eppendorf tubes with 100 μl 0.05 M Tris-HCl pH 7 on ice. The tubes were centrifuged ($15\,000\,g$ for 15 min at 4 °) and the supernatants recovered and frozen (-21 °) as 20 μl aliquots.

For quantitative studies of enzymatic activity, fungal material from the lower third of the fungus garden of *A. colombica* was sampled. The ants were removed and 361 mg of this material was extracted in 361 μl 0.05 M Tris-HCl pH 7. After grinding and centrifugation as described above, dilutions from 90 to 10% were prepared by adding ice-cold extraction buffer and stored as aliquots at -21 °.

Faecal droplets were obtained from medium sized workers (4–5 mm) recovered from the middle part of the fungus-garden of both ant-colonies. The ants were individually placed on a hydrophobic plastic sheet under a dissection microscope. Approximately one third of the ants released a faecal droplet, either as a result of being held by the legs with the forceps or by being gently squeezed on the head. The droplet volumes were determined to be 0.1–0.2 μl by comparison with pipetted water droplets on the same hydrophobic surface. This volume corresponds to the volume of the ant rectum (Martin & Martin 1970). The droplets were diluted with 4 μl distilled water and samples of 2 μl of this liquid were recovered with a 10 μl pipette-tip and stored at -21 °.

Electrophoresis and enzymatic assays

The proteins were separated by isoelectric focusing (IEF) in a pH gradient from 3–10 or 6–8 using the Mini IEF Cell (Bio-Rad Laboratories). Gels were cast as described in Skovgaard & Rosendahl (1998). Samples of 2 μl were loaded on an approximately 2 × 4 mm piece of Sample Applicator (Pharmacia Fine Chemicals AB, Uppsala) at equal distance from the electrodes. Focusing was carried out at constant voltage of 100 V for 15 min followed by 200 V for 15 min and 450 V for 60 min. Gels were removed immediately after focusing and covered with a pre-cast overlay. Overlays were cast in a 55 × 95 mm tray and consisted of 9 ml substrate solution, mixed with 2% w/v Agarose (AppliChem, Darmstadt) and heated in a microwave oven until dissolved.

We tested for specific enzyme activities with the following substrates: The pectinase substrate solution contained 0.66 g pectin (Sigma P-9135) and 20 mg CaCl (Merck 2387) dissolved in 100 ml McIlvaine's citric acid-phosphate buffer pH 5.0. The carboxymethylcellulase (CMCase) substrate solution contained 0.30 g carboxymethylcellulose (CMC) (Sigma C-5013) dissolved in 100 ml Sørensen's phosphate buffer pH 7.0. Laccase substrate solution was made by dissolving 0.055 g of the artificial laccase substrate ABTS (2.2′-Azino-di-(3-ethyl-benzthiazolin-sulfonate(6); Boehringer-Mannheim 1084220) in 5 ml $2\,g\,l^{-1}$ $K_2HPO_4 \cdot 3H_2O$, $2\,g\,l^{-1}$ KCL and $2\,g\,l^{-1}$ $MgSO_4 \cdot 7H_2O$ added to 100 ml 0.1 M Tris-HCl pH 7.1. The protease substrate solution contained 2.0 g Skim Milk (Difco) in 100 ml Sørensen's phosphate buffer pH 7.0.

The gels with overlays were incubated at 37 ° in a plastic-box with moist tissue paper to maintain humidity. ABTS overlays (laccase) were incubated for 45 min, pectin overlays for 1 h, skim milk overlays for $1\frac{1}{2}$ h and CMC overlays for 2 h.

After incubation, gels with pectin overlays were placed in an aqueous solution of 0.03% w/v Ruthenium Red (Sigma R-2751) at room temperature for one hour and de-stained in distilled water at 4 ° overnight. This assay for pectinases was modified from Cruickshank & Wade (1980) where pectin lyases produced yellow bands or clear bands with a yellow edge on the red background, while polygalacturonases produced pale or clear bands where the pectin had been degraded. In our study all bright bands showed yellow colorations and accordingly these bands were identified as pectin lyases. Dark red bands in the overlay were interpreted as evidence for pectin esterase activity, since de-esterification of pectin increases the number of sites stainable by ruthenium red (Sterling 1970). Pectin lyase bands were best resolved directly after the staining, whereas the pectin esterase bands appeared after the removal of excess dye.

The CMCase bands were visualized by the technique of Beguin (1983). The CMC-overlay was after incubation stained in 0.1% w/v aqueous solution of Congo red (Sigma C-6767) for 20 min, and excess dye was removed by transferring the overlay to an aqueous solution of 1 M NaCl at 4 ° overnight. The CMCase

Table 1. The 11 enzymes of the pure culture symbiotic fungi of *Acromyrmex echinatior* and *Atta colombica* detected in IEF gel overlays.

Enzyme		pI[b]
Pectin lyase	A[a]	5.0–5.1
	B	5.5–5.7
	C	6.2–6.3
	D	6.4–6.5
Pectin esterase	A	7.0–7.1
	B	7.6–7.8
CMCase	A	<4.45[c]
	B	6.0–6.4
Laccase	–	6.3–6.5
Protease	A	6.9–7.0
	B	9.4–9.6

[a] Letters indicate isoforms of the enzyme.

[b] Isoelectric point.

[c] The pI of CMCase A was out of range compared to the standard proteins.

Table 2. The number of faecal droplets of each ant species examined with the four different overlays.

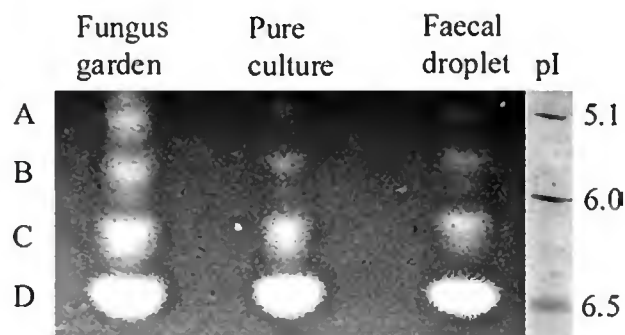| Type of overlay	*Acromyrmex echinatior*	*Atta colombica*
Pectin	14	12
CMC	2	2
ABTS	2	4
Skimmed milk	1	3

Fig. 1. The bands of pectin lyase activity. The enzymes were separated by IEF and diffused from their isoelectric point into the substrate overlay to produce hazy zones of activity. A–D, isoforms of pectin lyase. Samples contained extracts of garden material, extracts of pure cultures of the symbiotic fungus or faecal droplets of workers from the *Atta colombica*-colony. The stained marker-proteins and their pI values are given to the right.

activity appeared as clear bands in the red-orange overlay. The activity revealed by the CMC assay is generally interpreted as an endoglucanase, one of the components of the enzyme complex required to degrade cellulose (Eriksson, Blanchette & Ander 1990).

The skim milk overlay visualizes protease activity. Clear bands appeared in the white overlay when the milk proteins were degraded. The laccase bands appeared bright green in the otherwise clear ABTS overlay. Further characterization of the laccases in mycelial extracts was carried out with an assay using catechol as substrate (Madhusudhan, Taylor & Miles 1994). Following IEF, the gel was immersed in a solution of 10 mM Catechol (Sigma C-9510) and 10 mM L-proline (Merck 7434) and 100 mM Na_2HPO_4 pH 7 until a purple band appeared.

The isoelectric point (pI) of the bands in the overlays was determined by comparison with the bands of IEF standard proteins (Bio-Rad Laboratories, Richmond, CA) with pI 4.45–9.6.

Two µl samples of the dilution series of fungus garden extracts and samples of 2–2.4 µl of the non-diluted extract were submitted to IEF and staining in pectin overlays. The Region of Interest (ROI) feature of Kodak 1D Image Analysis Software was used to measure the net intensity of pectin lyase bands in digital images of the overlays. The net intensity of a band was defined as the sum of pixel-intensities within ellipse-shaped ROIs minus the background pixel intensity, which was set as the median pixel-value of the perimeter of the ROI.

A standard curve was made by plotting the intensities of the pectinases as a function of the amount of fungus garden extract loaded on the gels.

To overcome errors due to differences in staining between gels the values for the enzyme activities on each gel were divided by the activity of a 2 µl non-diluted sample on the same gel.

RESULTS

Eleven enzymes were detected in pure culture mycelia extracts of both symbiotic fungi (Table 1). 40 faecal droplets were examined with IEF and overlays (Table 2). In 37 of 40 faecal droplets the same enzymes were detected as in fungal mycelia and no additional enzymes were present, giving pictures as illustrated in Fig. 1. The remaining three faecal droplets were those from *Atta colombica* examined for proteases: In two droplets only protease A (which also had the strongest activity in the mycelial extract) appeared and in the third no enzyme activity could be detected. 11 fungal enzymes were detected in faecal droplets of *Acromyrmex echinatior* and ten fungal enzymes (protease B absent) were detected in faecal droplets of *Atta colombica*. The only enzymatic difference between the two ant species was the pI value of CMCase B. This was 6.0–6.2 and 6.2–6.4 for the symbiotic fungi and faecal droplets of *Acromyrmex echinatior* and *Atta colombica*, repectively.

The pure culture extract of the *Acromyrmex echinatior* fungus was examined with the assay for degradation of catechol. One band appeared and it had the same pI as the bands in the ABTS-overlay, which confirms that this enzyme is a laccase.

Fig. 2 shows the standard curve obtained for relative net intensity of pectin lyase D as a function of the amount of fungus garden extract applied to the gel.
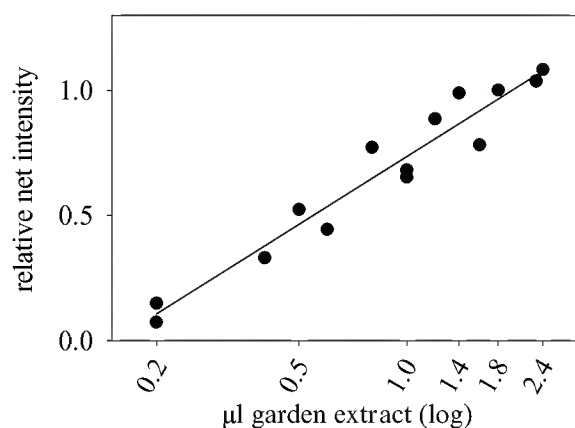
Fig. 2. The correlation between the relative net intensity of the bands of pectin lyase D and μl of fungus garden extract in the sample ($n = 14$; $r^2 = 0.94$). The samples of 2 μl non-diluted extract used for calibration are excluded.

The curve is based on the calibrated values from four gels and could be adequately described by a logarithmic function.

Six samples, each containing the equivalent of half a faecal droplet of *Atta colombica*, were submitted to IEF alongside the garden extract samples. The intensities of the pectin lyase D bands of the faecal samples were divided by the intensity of the band from 2 μl non-diluted garden extract. These calibrated values were all within the range of values of the standard curve (Fig. 2). The activity of pectin lyase D in the six faecal droplets could thus be estimated as equivalence fractions of μl garden extract, using 0.20 μl as a conservative estimate of the volume of a single average faecal droplet. One μl faecal droplet of *A. colombica* contained the same pectin lyase D activity as 10.2 μl garden extract on average, which correspond to the activity in 5.1 μg garden material (range of 1.4 to 9.7 μg).

DISCUSSION

This study adds to the knowledge of attine faecal enzymes by showing that the pectinases and CMCases have a fungal origin and by reporting the presence of fungal laccases. Boyd & Martin (1975a) previously purified one serine protease and two metallo-endoproteases of fungal origin from faecal droplets of *Atta texana*. The nature of the proteases A and B reported in our study, which could allow further comparison with these earlier results, remains to be investigated. However, the fungal origin of all hitherto investigated faecal enzymes indicate that the workers of leaf-cutting ants actively transfer digestive enzymes obtained from their symbiotic fungus to the youngest parts of their fungus garden.

The presence of active acquired enzymes in the gut does not necessarily prove that these enzymes increase the digestive efficiency of the animal partner (Kukor & Martin 1986) and similarly, the faecal enzymes of

leaf-cutting ants could just be traces of fungal enzymes that accidentally have escaped proteolysis in the gut and that do not contribute to the degradation of leaf material in the fungus garden. However, we found that the activity of a specific fungal enzyme was on average five times higher in the faecal droplets as in the mature garden material. Since fungal material only constitutes part of the worker-diet (Littledyke & Cherrett 1976, Quinlan & Cherrett 1979, Bass & Cherrett 1995), the high level of activity of the fungal enzymes in the faecal droplets can therefore not be explained as an insignificant remnant that failed to be digested.

More likely, the level of enzyme activity reflects a key role for acquired fungal enzymes in the maintenance of the fungus-garden. The mechanisms responsible for the apparent concentration of fungal enzymes in the gut are not known, but could simply be the absorption of water from the gut content. Moreover, to remain functional in faecal droplets, the fungal enzymes must be resistant to the proteolytic activity of the ant gut. Alternatively, the symbiotic fungus could concentrate the enzymes in specific parts of the mature fungus-garden preferably eaten by the ants, either in the gongylidia or, perhaps less likely, extracellularly. Regardless of the mechanism, the high level of enzyme activity in the faecal droplets as compared to the mature fungus garden indicates the importance of the transfer of fungal digestive enzymes within the fungus garden.

The actual functional roles of the transferred fungal enzymes in the fungus garden are unknown, but some reasonable inferences are possible. CMCases and pectinases could initiate hyphal colonization by attacking plant cell walls when a faecal droplet is placed on the finely fragmented leaf-material. The application of pectinases could be especially important to accelerate the early stages of degradation, due to its macerating effect on plant tissue (van Den Broek *et al.* 1997). The ant's own metabolism is less likely to benefit from pectinases and CMCase, since they do not ingest entire plant-cells, but only the sap (Febvay & Kermarrec 1981). Insects normally produce their own proteases, but the fungal proteases could supplement the digestion in some essential ways. As the degradation of leaf-material is most likely nitrogen limited (Poincelot 1974), a rapid mobilization of nitrogen from the new leaf-material by the faecal proteases may result in a faster subsequent degradation in the fungus-garden.

Another group of enzymes that may play an important role in the symbiosis are the laccases, or polyphenol oxidases, that catalyze the oxidation of phenolic substances (Thurston 1994). Several authors have suggested that the ability of leaf-cutting ants to exploit a wide range of plant species is based on the presence of fungal enzymes, including laccases, detoxifying the chemical defense substances of these plants (Cherrett 1980, Cherrett *et al.* 1989, Nichols-Orians 1991, Powell & Stradling 1991). According to this theory the acquisition of fungal laccase by the ants could facilitate the detoxification of ingested plant juices, making

the workers capable of exploiting plants that otherwise would be inedible or toxic. It has not yet been demonstrated that laccases from *Leucoagaricus gongylophorus* actually are detoxifying defensive plant compounds, but the presence of laccases in the leaf-cutting ant system underlines the need for further enzymatic studies to investigate the hypotheses of detoxification in the ant gut, as well as in the fungus garden.

The symbiotic fungi of the *Atta* and *Acromyrmex* leaf-cutting ants and of the two related higher attine genera, *Sericomyrmex* and *Trachymyrmex*, are all characterized by the formation of gongylidia (Mueller 2002). These four genera represent the most derived group within the *Attini* (Wetterer, Schultz & Meier 1998). The other seven (more basal) genera culture fungi that belong to *Leucoagaricus/Leucocoprinus* without gongylidia, except for a single genus, *Apterostigma*, where some species culture a tricholomataceaous fungus, which also lack gongylidia (Chapela *et al.* 1994, Moncalvo *et al.* 2002, Mueller 2002). These fungi have a less strict affinity to the symbiosis with ants, as a number of lineages are still closely related to free living strains (Mueller, Rehner & Schultz 1998). The lower attine ants that cultivate these fungi are also known to be opportunistic in their substrate-choice and to collect mainly dead material as substrate for their fungus gardens (Leal & Oliveira 2000, Currie 2001). Future studies should clarify whether acquisition and transfer of fungal enzymes is a fundamental and basal trait of the symbiosis, or an innovation realised by the common ancestor of all higher attines or by the leaf-cutting ants only, as an adaptation to their more specialised choice of live vegetable substrate.

REFERENCES

Bass, M. & Cherrett, J. M. (1995) Fungal hyphae as a source of nutrients for the leaf-cutting ant *Atta sexdens*. *Physiological Entomology* **20**: 1–6.

Beguin, P. (1983) Detection of cellulase activity in polyacrylamide gels using congo red stained agar replicas. *Analytical Biochemistry* **131**: 333–336.

Bot, A. N. M. & Boomsma, J. J. (1996) Variable metapleural gland size-allometries in *Acromyrmex* leafcutter ants (*Hymenoptera: Formicidae*). *Journal of the Kansas Entomological Society* **69** (Suppl.): 375–383.

Boyd, N. D. & Martin, M. M. (1975a) Fecal proteinases of the fungus growing ant *Atta texana*: properties, significance and possible origin. *Insect Biochemistry* **5**: 619–635.

Boyd, N. D. & Martin, M. M. (1975b) Fecal proteinases of the fungus growing ant *Atta texana*: their fungal origin and ecological significance. *Journal of Insect Physiology* **21**: 1815–1820.

Cazin, J. J., Wiemer, D. F. & Howard, J. J. (1989) Isolation, growth characteristics and long-term storage of fungi cultivated by attine ants. *Applied and Environmental Microbiology* **55**: 1346–1350.

Chapela, I. H., Rehner, S. A., Schultz, T. R. & Mueller, U. G. (1994) Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* **266**: 1691–1694.

Cherrett, J. M. (1980) Possible reasons for the mutualism between leaf-cutting ants (*Hymenoptera: Formicidae*) and their fungus. *Biologie-Ecologie Méditerranéenne* **7**: 113–122.

Cherrett, J. M., Powell, R. J. & Stradling, D. J. (1989) The mutualism between leaf-cutting ants and their fungus. In *Insect-fungus Interactions* (N. Wilding, N. M. Collins, P. M. Hammond & J. F. Webber, eds): 93–120. Academic Press, London.

Crnickshank, R. H. & Wade, G. C. (1980) Detection of pectic enzymes in pectin acrylamide gels. *Analytical Biochemistry* **107**: 177–181.

Currie, C. R. (2001) A community of ants, fungi, and bacteria: a multilateral approach to studying symbiosis. *Annual Review of Microbiology* **55**: 357–380.

Eriksson, K. E. L., Blanchette, R. A. & Ander, P. (1990) *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer Verlag, Berlin.

Febvay, G. & Kermarrec, A. (1981) Morphology and functioning of the infrabuccal filter in an attine *Acromyrmex octospinosus* (*Hymenoptera: Formicidae*): role of the infrabuccal pocket. *International Journal of Insect Morphology and Embryology* **10**: 441–450.

Fisher, P. J., Stradling, D. J. & Pegler, D. N. (1994) *Leucoagaricus* basidiomata from a live nest of the leaf-cutting ant *Atta cephalotes*. *Mycological Research* **98**: 884–888.

Kukor, J. J. & Martin, M. M. (1986) The effect of acquired microbial enzymes on assimilation efficiency in the common woodlouse *Tracheoniscus rathkei*. *Oecologia* **69**: 360–366.

Leal, I. R. & Oliveira, P. S. (2000) Foraging ecology of attine ants in a neotropical savanna: seasonal use of fungal substrate in the cerrado vegetation of Brazil. *Insectes Sociaux* **47**: 376–382.

Littledyke, M. & Cherrett, J. M. (1976) Direct ingestion of plant sap from cut leaves by the leaf-cutting ants *Atta cephalotes* and *Acromyrmex octospinosus Formicidae Attini*. *Bulletin of Entomological Research* **66**: 205–217.

Madhusudhan, V. V., Taylor, G. S. & Miles, P. W. (1994) The detection of salivary enzymes of phytophagous *Hemiptera*: a compilation of methods. *Annals of Applied Biology* **124**: 405–412.

Martin, J. S. & Martin, M. M. (1970) The presence of protease activity in the rectal fluid of attine ants. *Journal of Insect Physiology* **16**: 227–232.

Martin, M. M., Boyd, N. D., Gieselmann, M. J. & Silver, R. G. (1975) Activity of fecal fluid of a leaf cutting ant toward plant cell wall polysaccharides. *Journal of Insect Physiology* **21**: 1887–1892.

Martin, M. M., Gieselmann, M. J. & Martin, J. S. (1973) Rectal enzymes of attine ants. α-amylase and chitinase. *Journal of Insect Physiology* **19**: 1409–1416.

Moncalvo, J. M., Vilgalys, R., Redhead, S. A., Johnson, J. E., James, T. Y., Aime, M. C., Hofstetter, V., Verduin, S. J. W., Larsson, E., Baroni, T. J., Thorn, R. G., Jacobsson, S., Clemencon, H. & Miller, O. K. jr (2002) One hundred and seventeen clades of euagarics. *Molecular Phylogenetics and Evolution* **23**: 357–400.

Mueller, U. G. (2002) Ant versus fungus versus mutualism: ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. *American Naturalist* **160**: S67–S98.

Mueller, U. G., Rehner, S. A. & Schultz, T. R. (1998) The evolution of agriculture in ants. *Science* **281**: 2034–2038.

Nichols-Orians, C. M. (1991) Differential effects of condensed and hydrolyzable tannin on polyphenol oxidase activity of attine symbiotic fungus. *Journal of Chemical Ecology* **17**: 1811–1820.

Pagnocca, F. C., Bacci, M. jr, Fungaro, M. H., Bueno, O. C., Hebling, M. J., Sant, A. A. & Capelari, M. (2001) RAPD analysis of the sexual state and sterile mycelium of the fungus cultivated by the leaf-cutting ant *Acromyrmex hispidus fallax*. *Mycological Research* **105**: 173–176.

Poincelot, R. P. (1974) A scientific examination of the principles and practice of composting. *Compost Science* **15**: 24–31.

Powell, R. J. & Stradling, D. J. (1991) The selection and detoxification of plant material by fungus-growing ants. In *Ant-plant Interactions* (C. R. Huxley & D. F. Cutler, eds): 19–41. Oxford University Press, Oxford.

Quinlan, R. J. & Cherrett, J. M. (1979) The role of fungus in the diet of the leaf cutting ant *Atta cephalotes*. *Ecological Entomology* **4**: 151–160.

Skovgaard, K. & Rosendahl, S. (1998) Comparison of intra- and extracellular isozyme banding patterns of *Fusarium oxysporum*. *Mycological Research* **102**: 1077–1084.

Sterling, C. (1970) Crystal structure of ruthenium red and stereochemistry of its pectic stain. *American Journal of Botany* **57**: 172–175.

Thurston, C. F. (1994) The structure and function of fungal laccases. *Microbiology* **140**: 19–26.

van Den Broek, L. A. M., Den Aantrekker, E. D., Voragen, A. G. J., Beldman, G. & Vincken, J. P. (1997) Pectin lyase is a key enzyme in the maceration of potato tuber. *Journal of the Science of Food and Agriculture* **75**: 167–172.

Weber, N. A. (1972) The fungus culturing behavior of ants. *American Zoologist* **12**: 577–587.

Wetterer, J. K., Schultz, T. R. & Meier, R. (1998) Phylogeny of fungus-growing ants (Tribe Attini) based on mtDNA sequence and morphology. *Molecular Phylogenetics and Evolution* **9**: 42–47.

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