Ultrastructure of the Entomopathogenic Fungus Metarhizium anisopliae During Cuticle Penetration in the Locust, Locusta migratoria (Insecta: Acrididae)

Authors: Zhang, Xinyang, Yang, Yingyang, and Zhang, Long

Source: Journal of Orthoptera Research, 19(1) : 115-119

Published By: Orthopterists' Society

URL: https://doi.org/10.1665/034.019.0117

Ultrastructure of the entomopathogenic fungus Metarhizium anisopliae during cuticle penetration in the locust, Locusta migratoria (Insecta: Acrididae)

Submitted April 20, accepted June 16, 2010

XINYANG ZHANG, YINGYANG YANG, LONG ZHANG

Key Laboratory for Biological Control of Ministry of Agriculture, Department of Entomology, China Agricultural University, Beijing, 100193, China. Email: locust@cau.edu.cn

Abstract

Penetration of the cuticle of *Locusta migratoria* by the entomopathogenic fungus *Metarhizium anisopliae* was investigated using scanning and transmission electron microscopy. Scanning electron microscopy revealed that conidia of *M. anisopliae* attached mostly to the sockets of the setae and to the intersegmental membranes. Fourth instar nymphs of locusts treated with the fungus were maintained at 28°C. At this temperature, the fungal conidia germinated and produced appressoria on the cuticle of the locust within 1 day postinoculation. The penetrant structure entered directly through the epicuticle and exocuticle, with no lateral development. A fusiform penetrant structure then extended laterally between the layers of the endocuticle, with the spaces between the layers being enlarged due to lysis of the layers close to the penetrant. The penetrant structures exhibited various forms; most were fusiform, with many lysosomes and mitochondria, but no vacuoles. The penetrant hyphae ultimately invaded and colonized the haemocoele.

Key words

Locusta migratoria, *Metarhizium anisopliae*, penetrating process, cuticle, penetrant structure

Introduction

Locust outbreaks have been documented in Africa, North America, Asia, and Australia, and can be responsible for billions of dollars worth of agricultural losses (Prior *et al.* 1992). The oriental migratory locust *Locusta migratoria*, has been listed as one of the major pests of crops in China since 707 B.C. There is considerable interest in developing the entomopathogenic fungus *Metarhizium anisopliae* as an environmentally friendly alternative to chemical pesticides for the control of locusts (Zhang & Hunter 2005). Effective deployment of the fungus as a control agent will depend on a thorough understanding of the interactions between the fungal pathogen and its insect host, including the mechanisms of invasion and the development of the fungus within the host.

Structural studies of host-pathogen interactions have helped to improve our understanding of the invasion process of fungal pathogens, and helped in screening for more virulent strains. Entomopathogenic fungi penetrate the insect host's cuticle and grow rapidly in the host lymph, and the cuticle is therefore regarded as an important barrier to fungal infection (Leger 1991, Hajek & Leger 1994, Butt *et al.* 1995). Numerous studies have investigated the infection process demonstrated by the entomopathogenic fungus *M. anisopliae*, *e.g.*, *in vitro* production of appressoria, and germination and prepenetration events on the host cuticle (Zacharuk 1974, Hajek & Leger 1994, Leger 1991, Butt *et al.* 1995, Leger *et al.* 1989). Several microscopic studies of *M. anisopliae* infection of *Schistocerca gregaria* have been carried out (Gunnarsson 1988), with a focus on the haemocytic reaction. Hajek and Leger (1994) reviewed the interactions between insect hosts and fungal pathogens.

However, no detailed ultrastructural study has yet examined the penetration process of the entomopathogenic fungus *M. anisopliae*. This study therefore focused on the penetration of the *L. migratoria* cuticle by *M. anisopliae*. The results may help to reveal the mechanisms behind *M. anisopliae* infection and its development within the layers of the insect cuticle, as well as providing a potential means of screening fungal strains.

Materials and Methods

Maintenance of insect and fungal cultures.— *L. migratoria* were reared in the Department of Entomology, China Agricultural University (Beijing) at a temperature of 28 to 30°C, a relative humidity of 60% and under a photoperiod of 18:6 h (light:dark). Fresh wheat shoots were provided daily. Fourth instar nymphs were used in the experiments.

The isolate *M. anisopliae* var. *acridum* was used in this study and was kindly supplied by Becker Underwood, Australia. The product was stored as spore powder at 4°C until use.

Inoculation.—Approximately 20,000 *M. anisopliae* spores were applied to each locust nymph by placing a 2-µl dose of 1×10^8 conidia ml^{-1} in 0.03% Tween 80, onto the dorsal part of the abdomen using a pipetteman. The insects were then transferred to an illumination box at 28°C under a 18:6-h photoperiod in humid conditions (>80%), and supplied daily with fresh wheat seedlings.

Scanning electron microscopy (SEM).—Fourth-instar larvae inoculated with *M. anisopliae* were prepared for the SEM at 1, 2, 3, 4, and 5 d post-inoculation. The insects were chilled on ice. The infected area of the abdomen was then excised using a scalpel blade and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4, overnight, rinsed for 30 min in PBS (four changes), postfixed with 2% OsO_4 in 0.1 M PBS and dehydrated in an ethanol series. The specimens were critical-point dried, mounted on aluminum stubs and sputter-coated with gold/palladium (40:60) in a Polaron E5400 high-resolution sputter coater. The samples were examined using a Hitachi S570 SEM (Hitachi Ltd., Tokyo, Japan).

Transmission electron microscopy (TEM).—The cuticle from the dorsal part of the abdomen was excised and fixed at daily intervals after treatment (1, 2, 3, 4 and 5 d postinoculation), using the same process as for the SEM samples, with the addition of a final rinse

with 100% acetone. The fixed cuticle was embedded in Epon 618 and 0.500 to 0.6 µm ultrathin sections were cut using a glass knife on an LKB V Ultramicrotome (LKB Company, Bromma, Sweden). The sections were stained with uranyl acetate and lead citrate in a LKB ultrastainer, mounted on Formvar-coated grids, and examined using a Hitachi H-7500 TEM (Hitachi Ltd).

Results

Attachment and germination of fungal conidia.— Conidia of *M. anisopliae* were capable of binding to any site on the locust cuticle, but were frequently trapped by the setae (Fig. 1a). Many conidia also attached to the intersegmental folds, particularly to the intersegmental membranes, or to the tergum and sternum. Secretion of adhesive mucus from the conidia and the hyphae on the cuticle surface could be observed as the conidia swelled during pregermination.

By one day after inoculation, the conidia had germinated and started to grow on the surface of the cuticle. Most conidia produced a single short germ tube from their tip or middle region (Fig. 1b, c), but a few exhibited bipolar germination. The mode of infection of *M. anisopliae* on the host cuticle appeared to consist of production of a germination tube by the conidia (Fig. 1c), followed by production of an appressorium (Fig. 1d). The appressorium varied in morphology, including cupped, clavate, curly and globose-shaped structures, and ranged in diameter from 2-7 µm. All the infection structures combined with the host cuticle without producing any melanotic lesions.

Penetration of the locust cuticle.—Cross-sections of uninfected locust cuticle showed that it consisted of several layers (Fig. 2a): the epicuticle, the procuticle comprised of the exocuticle and endocuticle, and the innermost epidermis. The epicuticle was very thin (0.1-3 µm) but stained very darkly. The exocuticle beneath the epicuticle was thicker (3-5 µm), while the innermost layer, the endocuticle, was even thicker (8-12 µm). The endocuticle was composed of lamellae, each comprising a mass of microfibers arranged in sheets.

Examination of the ultrastructure of the infected cuticle at 2 days postinoculation, showed that the fungus initially penetrated across the exocuticle and then developed between the layers of the endocuticle (Fig. 2 a, b).

Penetration of the epicuticle was achieved via a narrow peg produced by an appressorium (Fig. 1c, d). The penetrant structures subsequently reached the endocuticle, where they developed laterally between the sheets of endocuticle (Fig. 2a, b). The shape of the penetrant structures varied at this stage, but were mainly comprised of fusiform cells (Fig. 2a-d). The cells typically had a clear, large nucleus, with high densities of mitochondria and lysosomes. The cells had unseen or very thin cell walls, no vacuoles and no septa (Fig. 2d). Several layers of endocuticle surrounding the penetration structure were deformed, and parts of the layers directly contacting the penetrating structure were lysed, with serious infections also causing lysis of neighboring layers (Fig. 2a-c). The spaces around the penetrant structure were enlarged. Three days after inoculation, the penetrant structures had started to invade the epidermis, haemocoele and other tissues.

In summary, the ultrastructural events associated with *M. anisopliae* infection demonstrated several stages (Fig. 4). After landing on the host, the conidia started to penetrate the epicuticle by forming an appressorium (stage 1). Tube-like penetrant structures then directly invaded the epicuticle and exocuticle, but did not develop there (stage 2). On reaching the endocuticle, however, the fusiform penetrant structures developed laterally, between the procuticle layers. The layers close to the penetrant structures were lysed. The fungus was more active at this stage, having a larger nucleolus and more mitochondria and lysosomes, as well as more electron-dense cytoplasm (stage 3). The fungus then penetrated the endocuticle and developed within the haemocoele (stage 4). The form of the fungus varied at each stage of the penetration process.

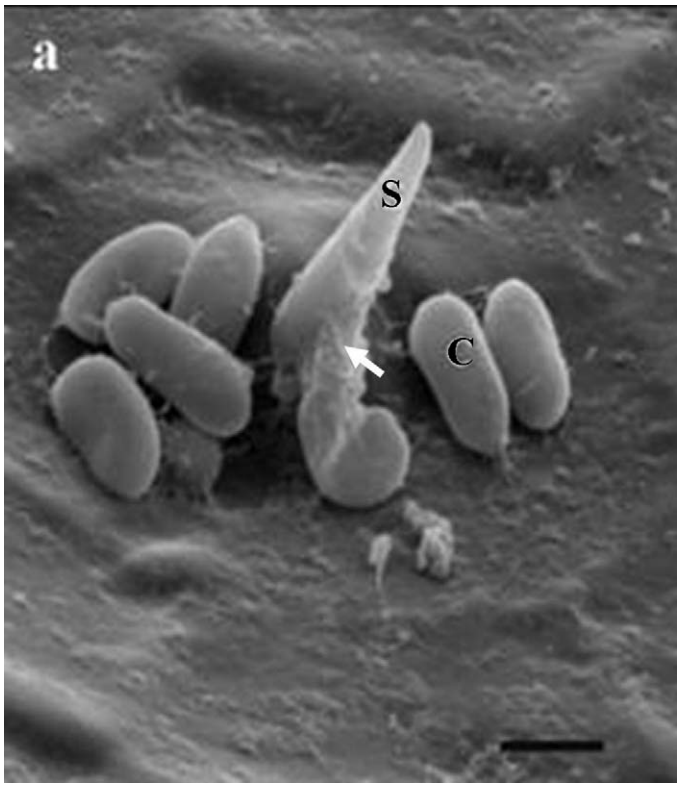
Discussion

Insect pathogenic fungi such as *M. anisopliae* invade their hosts directly through the external skeleton or cuticle (Jarrold *et al.* 2007). The cuticle appears to influence the adhesion, germination and appressorium differentiation of the fungus (Butt 1990). This represents one of the four stages of fungal penetration of the cuticle noted in the current study; conidia were most often observed at the bases of hair sockets or on the intersegmental membranes. The exocuticle is more highly sclerotized than other layers of the cuticle, and is therefore harder and more rigid; it is absent in areas of the integument where flexibility is required, *e.g.*, at joints and intersegmental membranes. These zones may be more suitable for attachment, recognition, germination and penetration of conidia.

However, the frequent penetration in these areas (Gunnarsson 1988, Vestergaard *et al.* 1999) may not be only a result of their structural weakness (Davies 1988); Wang and Leger (2005) found abundant appressorial formation by *M. anisopliae* conidia placed against plastic coated with locust-cuticle extract, indicating the importance of chemical factors in spore germination on the host cuticle. Pedrini *et al.* (2007) suggest that the surface structure, as well as the chemical composition of the cuticle, may be important. The cuticle contains numerous microenvironments within its folds and cleavages where humidity and temperature regimes may be more conducive to fungal germination and growth (Bidochka *et al.* 1997). The outermost epicuticular surface layer of the locust consists of complex polar and nonpolar lipids, and the fungus can use a wide range of aromatic organic compounds as nutrients during germination and other prepenetrant stages of growth (Jarrold *et al.* 2007). The absence of mechanical lesions on the cuticle suggests that the interaction between *M. anisopliae* and its host occurs via chemical dissolution of structures, rather than by purely mechanical breaching of tissues.

Activated fungal molecules can generally be classified as penetration effectors (toxins, enzymes) that reduce host defense, transporters that protect the fungus from host defenses, or components of signal transduction pathways that are required for fungal sensing of the host environment (Pedrini *et al.* 2007). However, host specificity is determined by the chemical signals utilized by the conidia to recognize their host and to promote germination on the host cuticle (Hall & Papierok 1982). Host specificity means that a fungus should have specific receptor systems to recognize their host's signals. The results of this study imply that the bases of hair sockets and the intersegmental membranes contained higher concentrations of the chemical signals recognized by the fungus.

Entomopathogenic fungi infect insects by breaching the host cuticle, which represents an important barrier to fungal infection (Leger 1991, Butt *et al.* 1995). The observations in this study showed that although the fungal tube-like penetrant structures directly penetrated the epicuticle and exocuticle, they never developed there, suggesting that locust epicuticle and exocuticle present more significant barriers to the fungus than endocuticle, even though this layer contains many chitin fiber sheets (Hajek & Leger 1994).



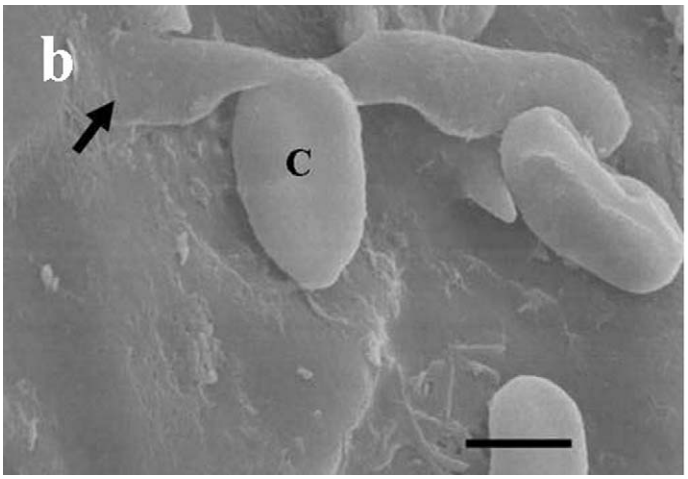
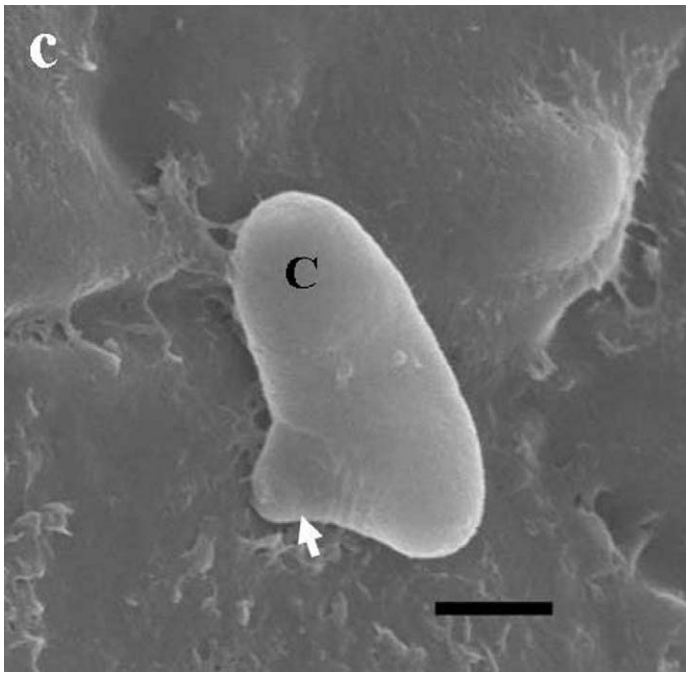
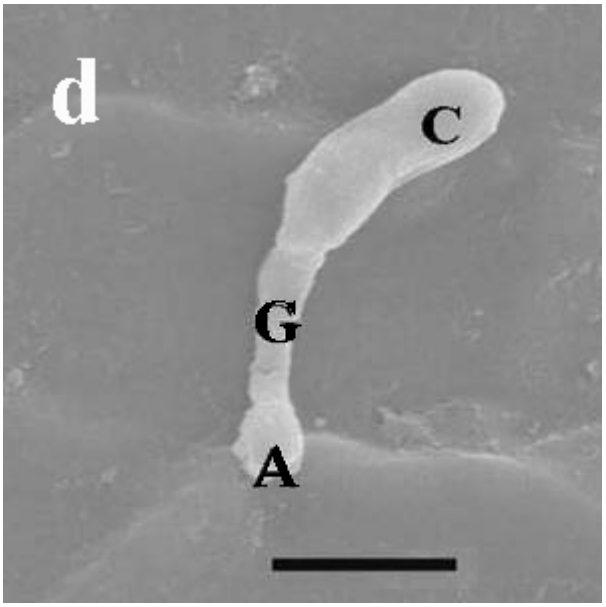


Fig. 1. Conidia of *M. anisopliae* and their germ tube and appressorium on locust cuticle, observed by scanning electron microscopy; ultrastructure of conidium with germination tube observed by transmission electron microscopy. **a.** Conidia (C) of *M. anisopliae* with mucus surrounding the sensillum (S). Scale bar, 4 µm. **b.** *Metarhizium* is inserting into the cuticle (arrow). Scale bar, 4.5 µm. **c.** Germ tube (arrow) produced from the middle of a conidium. Scale bar, 2 µm. **d.** An appressorium (A) at the end of a short germ tube (G), C, conidium. Scale bar, 5 µm.

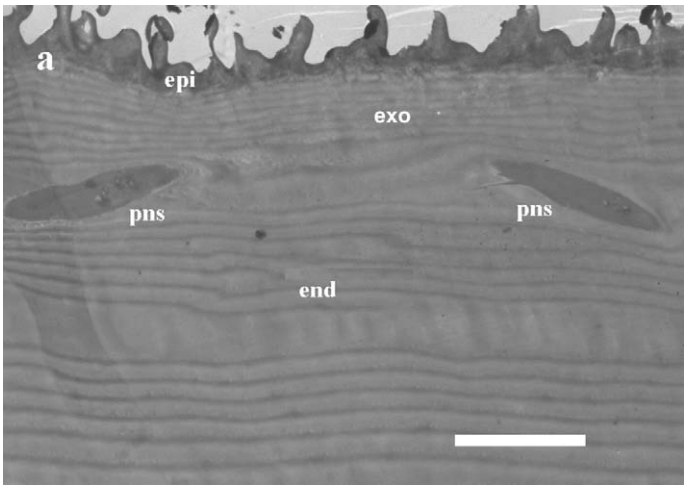




Once the fusiform penetrant structures reached the endocuticle, they then developed laterally between the layers, causing lysis of the layers close to the penetrating structures. Increased activity of the fungus at this stage was suggested by the presence of a larger nucleolus, more mitochondria and lysosomes, and more electron-dense cytoplasm. The penetrant structures were able to separate the layers of the locust, and their lateral expansion, together with the holes made in the cuticle, may weaken cuticle integrity and so facilitate further penetration (Brey *et al.* 1986) and dispersal of the pathogen's cuticle-degrading enzymes (Goettel *et al.* 1989).

Observations in this study suggest that, once the fungus has penetrated the endocuticle, it is able to develop rapidly within the haemocoele, soon leading to the death of the host. Damage to host structures, as well as nutrient exhaustion and toxicosis, are all likely to be contributory factors to host mortality (Gillespie & Claydon 1989).

Entomopathogenic fungi invoke adaptive biochemical processes and cellular differentiation within their host, resulting in the formation of a series of specific morphological structures that favor fungal development (Hajek & Leger 1994). The current observations identified several ultrastructures during the four stages of *M. anisopliae* penetration of the locust cuticle: the germination tube and appressorium on the surface of the cuticle, the tube-like



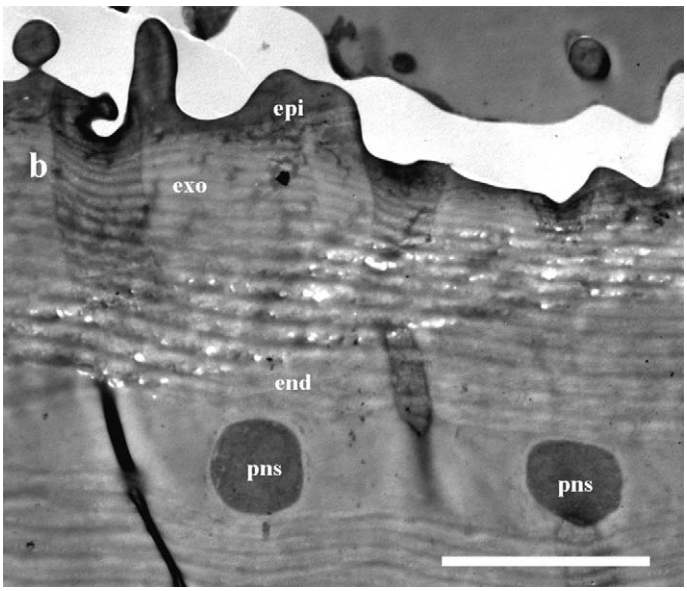
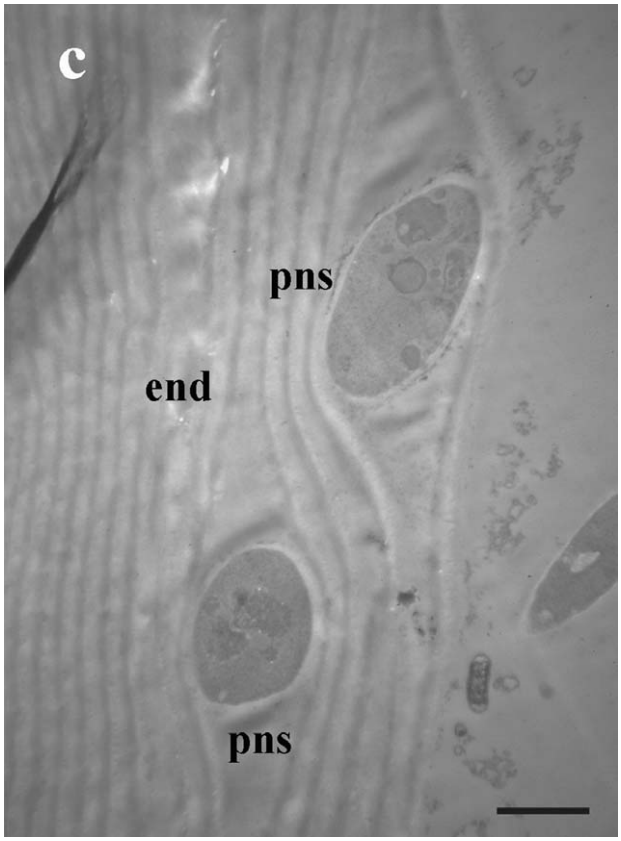
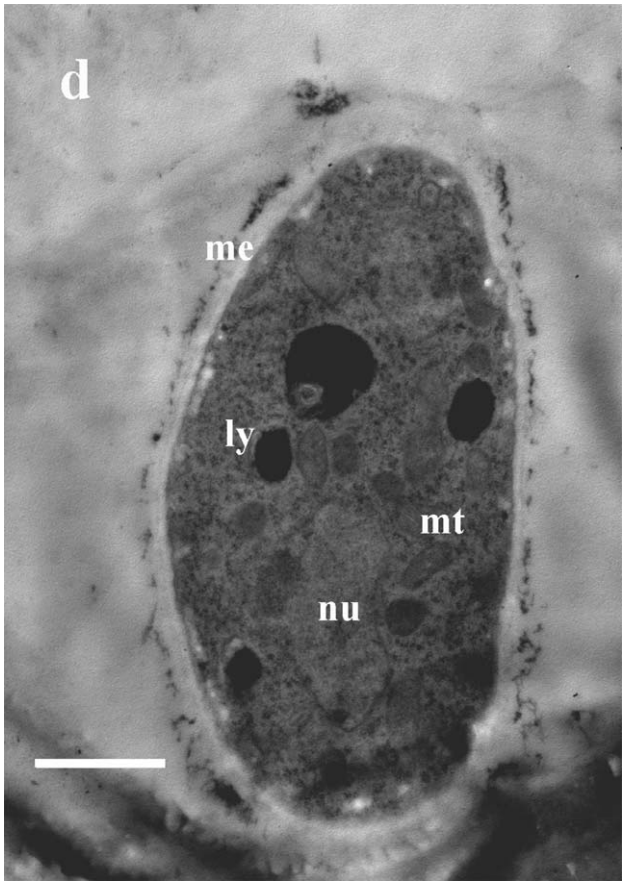


Fig. 2. The ultrastructure of *M. anisopliae* penetrant structures and locust cuticle during penetration process. **a**. Appearance in longitudinal section of penetrant strucuture of *M. anisopliae* in layers of locust cuticle. epi, epicuticle; exo, exocuticle; end, endocuticle; pns: longitudinal sections of fusiform penetration structures extend laterally between sheets of endocuticle. Scale bar, 5μm. **b.** Transverse section of the fusiform penetrant structures in locust cuticle. The penetrant structure had developed near to epidermis still in uninfected condition. pns, fusiform penetrant structure; exo, exocuticle; end, endocuticle. Scale bar, 5μm. **c.** The locust cuticle layers around penetrant structures were degraded. Scale bar 2μm. **d.** Ultrastructure of fusiform penetrant structure in cuticle. me, membrane of the cell; nu, nucleus; ly, lysosome; mt, mitochondrion. Scale bar, 1μm.





penetrant structure in the epicuticle and exocuticle, the fusiform penetrant structure in the endocuticle, and the hyphae in the haemocoele. It is possible that these forms of the penetrant structure of *M. anisopliae* may evolve from relationships between the pathogen and its host. Our study partially revealed ultratructural events of fungal pathogen-insect host interactions during cuticle penetration, that should help in screening for more virulent strains to control locusts. In our experimental results, we did find that the forms of the fungus in different layers of locust cuticle present differently.

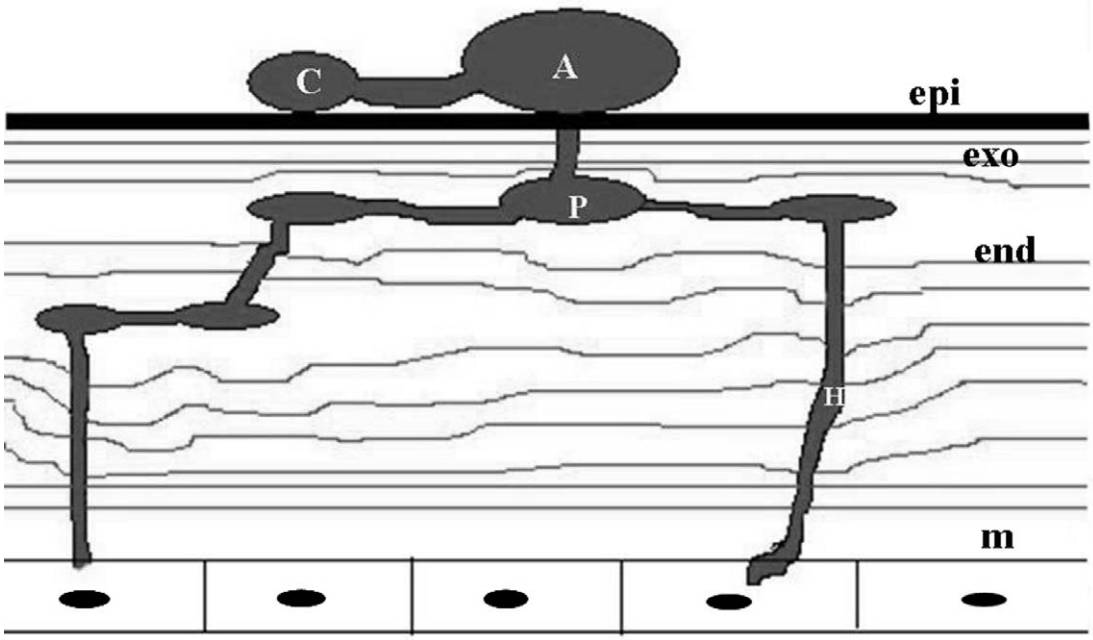


Fig. 3. Schematic representation of infection structures of *M. anisopliae* and cuticular change in transverse section of locust cuticle. C, condium; A, appressorium; P, penetrant structure; H, hypha; epi, epicuticle; exo, exocuticle; end, endocuticle; m, epidermis.

We did not find the form taken by the fungus when penetrating epicuticle: it should be studied further.

Acknowledgements

We thank Dr. D. Hunter for critical comments and manuscript revision. The research was supported by grants (2006AA10Z235) from the Ministry of Science and Technology, and a grant from the Ministry of Agriculture (200903021).

References

Bidochka M. J., Leger R.J., Roberts D.W. 1997. Mechanisms of deuteromycete fungal infections in grasshoppers and locusts: an overview. Memoires Entomological Society of Canada 171: 213-224.

Brey P.T., Latgé J.-P., Prevost M.C. 1986. Integumental penetration of the pea aphid, *Acyrthosiphon pisum* by *Conidiobolus obscurus*. Journal Invertebrate Pathology 48: 34-41.

Butt T. M. 1990. Fungal infection processes-a mini-review, pp. 121-124. Vth International Colloquium on Invertebrate Pathology, Adelaide, Society for Invertebrate Pathology: Adelaide..

Butt T.M., Ibrahim L., Clark S.J., Beckett A. 1995. The germination behaviour of *Metarhizium anisopliae* on the surface of aphid and flea beetle cuticles. Mycological Research 99: 945-950.

Davies R.G. 1988. Outlines of Entomology. Chapman and Hall, London.

Gillespie A.T., Claydon N. 1989. The use of entomogenous fungi for pest control and the role of toxins in pathogenesis. Pesticide Science 27: 203-215.

Goettel M.S., Leger R.J., Rizzo N.W., Staples R.C., Roberts D.W. 1989. Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. Journal Gen. Microbiology 135: 2233-2239.

Gunnarsson S.G.S. 1988. Infection of *Schistocerca gregaria* by the fungus *Metarhizium anisopliae*: cellular reactions in the integument studied by scanning electron and light microscopy. Journal Invertebrate Pathology 52: 9-17.

Hajek A.E., Leger R.J. 1994. Interactions between fungal pathogens and insect hosts. Annual Review Entomology 39: 293–322.

Hall R.A., Papierok B. 1982. Fungi as biological control agents of arthropods of agricultural and medical importance. Parasitology 84: 205-240.

Jarrold S.L., Moore D., Potter D., Charnley A.K. 2007. The contribution of surface waxes to pre-penetration growth of an entomopathogenic fungus on host cuticle. Mycological Research III: 240-249.

Leger R.J., Butt T.M., Goettel M.S., Staples R.C., Roberts D.W. 1989. Production *in vitro* of appressoria by the entomopathogenic fungus *Metarhizium anisopliae*. Experimental Mycology 13: 274-288.

Leger R. J. 1991. Integument as a barrier to microbial infections, pp. 284–306. In: Binnington K., Retnakaran A. (Eds). Physiology of the Insect Epidermis. CSIRO, Melbourne, Australia.

Pedrini N., Crespo R., Juárez M.P. 2007. Biochemistry of insect epicuticle degradation by entomopathogenic fungi. Comparative Biochemistry and Physiology 146: 124-137.

Prior C., Lomer C.J., Herren H., Paraiso A., Kooyman C., Smit J.J. 1992. The IIBC/IITA/DFPV collaborative research programme on the biological control of locusts and grasshoppers, pp. 394. C.J. Lomer, C. Prior (Eds), Biological Control of Locusts and Grasshoppers. CAB International. UK.

Vestergaard S., Butt T.M., Bresciani J., Gillespie A.T., Eilenberg J. 1999. Light and electron microscopy studies of the infection of the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) by the entomopathogenic fungus *Metarhizium anisopliae*. Journal Invertebrate Pathology 73: 25-33.

Wang C.S., Leger R.J. 2005. Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. Eukaryot cell. 4: 937-94.

Zacharuk R.Y. 1974. Ultrastructural pathology of the epidermis of molting elaterid larvae (Coleoptera) with a fungus and a bacterium in the ecdysial space. Journal of invertebrate pathology 23: 13-21.

Zhang L., Hunter D.M. 2005. Laboratory and field trials of Green Guard (*Metarhizium anisopliae* var. *acridum*) (Deuteromycotina: Hyphomycetes) against the oriental migratory locust (*Locusta migratoria manilensis*) (Orthoptera: Acrididae) in China. Journal of Orthoptera Research 14: 27-30.