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The contribution of surface waxes to pre-penetration growth of an entomopathogenic fungus on host cuticle

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ABSTRACT

A locust wing bioassay, that allowed an entomopathogenic fungus to be removed from host cuticle before penetration, was used to investigate the role of surface lipids and waxes in pre-penetration growth of the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. SEM and atomic force electron microscopy showed the impact of the fungus on the architecture of the cuticle surface. Although the fungus can germinate on authentic alkanes as the sole carbon source, only low levels of germination occurred on crude, non-polar wing cuticle extracts, containing a mixture of long-chain *n*-alkanes and other waxes (identified in particular by gas chromatography and mass spectroscopy). The fungus removed a large proportion of non-polar and polar components during pre-penetration growth on the wing. Polar crude extracts from *Schistocerca gregaria* hindwings, which contained fatty acids, fatty acid esters, glucose, amino acids and peptides, were strong promoters of germination, and poor germination was observed on a locust hindwing from which the extract had been taken. Thus simple polar compounds, also present on the surface, may be required to stimulate germination before the fungus can make use of a complex mixture of non-polar lipids.

Introduction

The role of host surface lipids and waxes in fungal pathogenesis of insects is poorly understood. We investigated the interaction between the desert locust, *Schistocerca gregaria*, and its specific fungal pathogen, the mitosporic, haploid entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* IMI 330189 (*Hypocreales*: *Clavicipitaceae*). However, our methodology, which can be applied to organisms other than insects, may also be useful for studies on other host–fungus interactions.

Insect pathogenic fungi like *M. anisopliae* invade their hosts directly through the external skeleton or cuticle. A considerable amount of research has been carried out on fungal enzymes that hydrolyse protein and chitin. These are the key components of the procuticle that comprises the bulk of the cuticle (Charnley 2003). However, the interaction of fungi with the cement layer and wax that cover the epicuticle, the thin outer layer of the cuticle (1–3 μm), has not been considered systematically. Although this is perhaps understandable, because of the complex chemistry of this section of the cuticle, it is also an omission because the epicuticle is the first point of contact between host and pathogen.

The major constituents of the cement layer are hydrocarbons with some tanned protein and lipids (Neville 1975). The wax layer (underneath the cement) in locusts comprises

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80 % hydrocarbons, and small amounts of esters, free fatty acids, free primary alcohols, and possibly some triacyglycerols (Lockey 1980; Lockey & Oraha 1990; Oraha & Lockey 1990). These and other studies on the surface cuticle of acridids have focussed exclusively on the dominant apolar constituents. However, Woods & Grula (1984) showed amino acids and glucosamine on the surface of larvae of the lepidopteran *Heliothis zea*, which supported growth of the entomopathogen *Beauveria bassiana*.

A variety of studies have shown that epicuticular lipids (extracted or as pure authentic compounds) may promote or inhibit fungal germination and/or growth. St Leger *et al.* (1988) showed that *M. anisopliae* var. *anisopliae* ARSEF 2575 (=ME1) can grow *in vitro* on authentic, single long-chain hydrocarbons. Extracted cuticle lipids, particularly straight-chain saturated fatty acids such as caprylic and capric acid, are inhibitory to fungal germination (Smith & Grula 1982; Koidsumi 1957), while linoleic acid is stimulatory (Barnes & Moore 1997). Sosa-Gomez *et al.* (1997) showed that hydrocarbons were binding substrates for *M. anisopliae* but not nutritional. Cuticle lipids from whiteflies, *Bemisia argentifolii*, were found to be inhibitory to the germination of *B. bassiana* and *Paecilomyces fumosoroseus* (James *et al.* 2003).

Lecuona *et al.* (1991) investigated the importance of the epicuticle as a source of nutrition for *B. bassiana*, during infection of *Ostrinia nubilalis* and *Melolontha melolontha*, by comparing the hydrocarbon profiles of extracts from control and fungus-treated insects. A broad-ranging reduction in cuticle hydrocarbons occurred during early mycosis. However, the preparations included fungus and insect material, and therefore the origin of particular lipids could not be determined with certainty.

In the present work a multifaceted approach has been taken to determine the role of cuticle surface chemicals in the pre-penetration growth of the locust pathogen *M. anisopliae* var. *acridum* IMI 330189. We used detached wings as an experimental tool because (1) being translucent they made light microscopy easier; (2) the wing is essentially no more than two layers of cuticle with a minimum of other material (in particular no fat body) (Uvarov 1966) that might otherwise contaminate lipoidal extracts; (3) the epicuticular lipid chemistry of the wing cuticle is the same as that of the body wall cuticle (Lockey & Oraha 1990), and (4) we have developed a protocol that enables us to remove the fungus from the wing before penetration and thus to determine, for the first time, the impact of the fungus on the chemistry and ultrastructure of the cuticle. This method is based on the observation that the extracellular matrices (mucus), produced by many fungi, including *M. anisopliae* (e.g., Zacharuk (1970), to aid adhesion to the host during pre-penetration growth, often comprise glycoproteins with a substantial β-1,3-glucan component (e.g., Latgé *et al.* 1987; Stahlmann *et al.* 1992; Carzaniga *et al.* 2001). Enzymatic hydrolysis of the β-1,3-glucan should weaken the mucus and make the fungus easy to dislodge.

The wing may not just be a useful experimental tool but have more direct relevance to pathogenicity. Seyoum (1994) showed that 51 % of an oil formulation of conidia of *M. anisopliae* var. *acridum*, applied to flying locusts by an ultra low volume sprayer, landed on the wings. James (1995) established that penetrant hyphae grow and spread between the bilayer of upper and lower wing cuticles. Therefore potentially the fungus could invade the body of the insect from the wing via the system of fine veins (hollow tubes of sclerotic, chitinous cuticle) that carry haemolymph, nerves and tracheae to and from the haemocoel.

Materials and methods

Fungal culture

The fungus, *Metarhizium anisopliae* var. *acridum* isolate IMI 330189 (CABI Bioscience, Egham), was stored as conidial suspensions in 10 % glycerol at −70 °C. Stock cultures were grown on quarter strength Sabouraud's dextrose agar (SDA) at 27 °C in the dark. The stocks were used for two to three months as sources of inocula for cultures grown on similar media that provided the conidia used in experiments. In most cases conidial suspensions were prepared in sterilized distilled water (dH_2O) from 14 d old cultures, as surfactants such as 0.04 % Tween 80 supported a low level of germination. Conidia were washed twice with aliquots of sterilized dH_2O; each time conidia were pelleted by centrifugation (5 min at 2500 ***g***) and the supernatant decanted. Conidia were finally suspended in fresh dH_2O to the required concentration. Suspensions of conidia were placed in a sonicating water-bath (MSE) at 15 °C for 20 min to prevent clumping.

Insect culture

Desert locusts, *Schistocerca gregaria* (*Orthoptera*: *Acrididae*), were reared on a diet of fresh wheat seedlings and bran supplemented with dried yeast. Blowflies, *Calliphora vomitoria*, were bought as pupae from a supplier and reared as adults on sugar and water. Flour beetles, *Tenebrio molitor*, were reared as described previously (Abboud *et al.* 1983). All insects were maintained at 28 °C under a 12 h daylight/12 h dark cycle.

Wing bioassay

Hindwings were detached from live, 10 d old adult desert locusts, beetles or flies and used directly, after sterilization in an atmosphere of propylene oxide or other treatment as indicated. Two or three wings were spread flat on 2 % water agar plates and inoculated with 1 ml of a $1 \times 10^6 - 4 \times 10^6$ conidia ml^{-1} in dH_2O, using a Potter spray tower (Burkard, Rickmansworth) and incubated at 27 °C and 100 % humidity. Germination and appressorial counts were made on pieces cut from the centre of the wing (to avoid edge effects), mounted on a microscope slide, stained with 0.1 % cotton blue in lactophenol and covered with a cover slip. Only conidia on the membrane were assessed, not those on veins. Germination was judged to have occurred when a germ tube was as long as it was broad. An appressorium was defined as an apical swelling on the germ tube of similar size to a conidium.

In vitro assays

On SDA. Plates containing quarter strength SDA were inoculated with a 1×10^6 ml^{-1} conidia in dH_2O, using a Potter spray tower (Burkhard) and then incubated in the dark at 27 °C.