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Comparison of bioassay responses to the potential fungal biopesticide *Metarhizium anisopliae* in *Rhipicephalus(Boophilus) microplus* and *Lucilia cuprina*

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ABSTRACT

Quantal response bioassays were conducted with cattle ticks and sheep blowflies with three different isolates of *Metarhizium anisopliae* and different methods of inoculation. Ticks were either topically dosed with 2 μl or immersed in the conidial preparations. Blowflies were either topically dosed with 2 μl of the conidial preparation or fed on conidia mixed with sugar. Probit analyses were carried out on the mortality data to compare the virulence of these isolates to ticks and blowflies and look for indications of different virulence mechanisms employed by *M. anisopliae* isolates when invading these hosts. One isolate (ARIM16) showed high virulence to both hosts killing 95% of ticks after 2 days and 88 (±2)% of blowflies after 4 days. Strikingly different mortality patterns indicated that virulence is dependent on different mechanisms in ticks and blowflies. The pattern of mortality seen with ticks suggested that the number of conidia adhering per unit area of the cuticle was more important for rapid tick death than the total number of conidia contacting the entire tick surface. Blowflies fed conidia mixed with food died rapidly after an initial lag phase regardless of dose.

1. Introduction

Observations made in the course of research into the potential of fungal biopesticides for livestock ectoparasites suggested variations in the way strains of *Metarhizium anisopliae* invaded cattle ticks (*Rhipicephalus microplus*) and sheep blowflies (*Lucilia cuprina*) (Leemon unpublished). These personal observations indicated two quite different patterns of invasion leading to death: Ticks treated with a suspension of *M. anisopliae* conidia develop opaque areas on their cuticles. The cuticle weakens as these coalesce and may rupture if handled. Death usually occurs when the cuticle has weakened and the opaque area covers at least half of either the dorsal or ventral surface, often in less than 48 h under optimal conditions of temperature and moisture. Fungal hyphae proliferate across the tick surface in concert with the fungal invasion. After death the fungal growth rapidly covers the entire tick surface and then sporulates. Sometimes fluids leak through the weakened cuticles. This pattern of fungal invasion of ticks appears to differ in severity between different *M. anisopliae* isolates. Some isolates which have been observed to take longer to kill ticks do not seem to cause the cuticle weakness. Blowflies exposed to *M. anisopliae* conidia either applied to their surface or added to their food seem to take longer to die than ticks when equivalent doses are used. After death the flies become mummified with densely packed mycelium inside their cavities. Fungal hyphae emerge from the cadavers and become visible on the exterior only when dead flies are exposed to moist conditions.

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Kershaw et al. (1999) proposed that two different virulence mechanisms are active when isolates of *M. anisopliae* invade and kill insects: a toxin strategy and a growth strategy. With the toxin strategy insects are killed by toxins such as the destruxins produced by some isolates of *M. anisopliae*; death is followed by the growth of hyphae throughout the insect body cavity. With the growth strategy insects are killed when fungal hyphae proliferate through the haemocoel using up nutrients. Leemon and Jonsson (2008) proposed a third virulence mechanism occurring when *M. anisopliae* invades ticks, an integument breakdown strategy. Ticks belong to a different class of arthropods to insects, and their integument has a different structure and composition from that of insects (Chapman, 1969; Sonenshine, 1991). It is possible that different mechanisms of virulence result from the actions of the extracellular enzymes produced by *M. anisopliae* on the integuments of ticks and blowflies.

An understanding of these perceived differences in tick and blowfly invasion and how they might occur could provide information to aid the application and formulation of fungal biopesticides to enhance their efficacy against either ticks or blowflies. One approach is to investigate differences in mortality patterns is to conduct probit analyses on the data from quantal response bioassays. Finney (1971) defines a bioassay (or biological assay) as the measurement of the potency of any stimulus by means of the response that it produces in living matter. He further defines a quantal response as an "all or nothing" response to a stimulus, where death is usually the measured response. In the present study ticks and blowflies were exposed to varying doses of fungal conidia from candidate isolates and the time to death recorded. Probit analysis is a convenient mathematical device that gives a diagrammatic representation of the dose–response relationship and provides estimates of the doses needed to achieve given mortality rates (Finney, 1971). The data are transformed depending on the distribution of the response data to get the best fit for a straight line. This line describes the relationship between dose and time to death (known as lethal time or LT). The most virulent isolate towards a given host can be established by comparing the time taken by a given dose to kill 50% (LT_{50}) of the test species. For groups in the same experiment the probit analysis also allows differences in slopes to be tested for statistical significance. A significant difference between slopes is considered to be likely to indicate qualitatively different mechanisms of mortality (Robertson et al., 2007).

The aim of these investigations was to compare the virulence of three isolates of *M. anisopliae* towards ticks and blowflies by conducting probit analyses on bioassay mortality data. By comparing slopes of the fitted lines the probit analyses will also test for indications of differences in the virulence mechanisms of the different isolates when invading blowflies and ticks. The three *M. anisopliae* isolates chosen from this investigation were ARIM10; ARIM16 and FI1218. When screening isolates against blowflies in previous research, ARIM10 was found to be one of the most virulent isolates, while ARIM16 was found to be one of the most virulent isolates screened against ticks. FI1218, a commercial isolate (BioBlast), was used for comparison.

Table 1
Actual spore concentrations in treatment doses.

Conidial dose	ARIM16 Actual (sp/ml)	FI1218 Actual (sp/ml)	ARIM10 Actual (sp/ml)
0	0	0	0
1	5×10^3	5×10^3	1×10^4
2	5×10^4	5×10^4	1×10^5
3	5×10^5	6×10^5	1×10^6
4	5×10^6	6×10^6	1×10^7
5	5×10^7	1×10^8	1×10^8
6	5×10^8	1×10^9	1×10^9

2. Materials and methods

2.1. Fungal inoculum

The *M. anisopliae* isolates ARIM10 and ARIM16 were originally isolated from soils in Queensland, Australia (respectively South Johnston in Nth Qld and Aratula in SE Qld). The isolate FI 1218 was obtained from Dr Richard Milner at CSIRO, Canberra. The fungal isolates were maintained on potato dextrose agar (PDA) slopes held at 4 °C and −20 °C. Fresh cultures initiated from these were maintained on both PDA and Sabouraud Dextrose Agar with 1% malt extract (SDAM) plates at 25 °C (Goettel and Inglis, 1997). Conidia were produced on solid rice media as described by Goettel (1984). The rice was dried at 20 °C then harvested by shaking through 300 μm and 150 μm Endicott sieves. Harvested spores were stored at 4 °C.

For each isolate 1.25 g of dried conidia were added to 50 ml of sterile 0.1% Tween 80. These solutions were then serially diluted 1:10 to give 6 conidial dilutions with approximate concentrations of 1×10^9 to 1×10^4 conidia/ml. The concentrations of the solutions were checked using a Neubauer haemocytometer (Goettel and Inglis, 1997). Conidial counts showed that the administered concentrations varied from the intended concentrations for the three *M. anisopliae* isolates (Table 1). Ticks and blowflies were inoculated with these 6 conidial doses and a control using the sterile 0.1% Tween 80 carrier. Conidial powder of the three isolates was also mixed with granulated sucrose in the following ratios: conidia (g):sucrose (g) – 0.035:3.5; 0.05:3.5; 0.25:3.5; 0.5:3.5 for blowfly self inoculation.

2.2. Ticks

Two experiments were conducted in which fully engorged female *R. microplus* ticks were either topically dosed with 2 μl of the inoculum or fully immersed in the inoculum. Ticks were obtained from the Animal Research Institute tick culture. In each trial 60 ticks each received one of 7 treatments with each fungal isolate. These treatments consisted of the 6 different conidial concentrations and the carrier. An untreated control group was also used for each fungal isolate. In the first trial 2 μl of inoculum was applied to the mid-dorsal surface of ticks. In the second trial ticks were immersed for 1.5 min in 10 ml of inoculum then blotted to remove excess liquid. After treatment ticks were placed singly in wells of 24 well micro-titre trays (Sarstedt) for incubation at 28 ± 0.5 °C in the dark, with 20 ticks per tray and three trays per treatment. Each trial involved