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Sweetpotato weevil, *Cylas formicarius* (Fab.) (Coleoptera: Brentidae) avoids its host plant when a virulent *Metarhizium anisopliae* isolate is present

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ABSTRACT

Metarhizium anisopliae has a wide range of coleopteran hosts, including weevils. Some susceptible insects are known to modify their behavior to prevent infection, typically detecting virulent strains by olfaction, and avoiding physical contact with sources of infection. Laboratory olfactometer assays were conducted on the sweetpotato weevil *Cylas formicarius* to test the hypothesis that insects would avoid a more virulent strain of *M. anisopliae* when presented with a strain of low virulence or an untreated control. When adult weevils were allowed to choose between paired test arenas containing sweetpotato roots and *M. anisopliae* isolates on agar cores, weevils avoided arenas with the highly virulent isolate QS155, showing a preference for either roots with uninoculated agar cores or cores with the low virulence isolate QS002-3. When roots or whole sweetpotato plants were inoculated with *M. anisopliae*, the preferences of weevils remained broadly similar; weevils were repelled by the highly virulent isolate QS155 when tested against either QS002-3 or uninoculated roots and plants, however weevils were not repelled by the low virulence isolate QS002-3 tested against uninoculated controls. When single-sex groups of weevils were tested separately in the olfactometer using uninoculated whole plants and plants treated with isolate QS155, males and females responded similarly and statistically identical preferences were found for the untreated plants. When weevils were released singly at different times of the day the response time for males was significantly shorter in the afternoon compared to the morning. Males were always significantly faster to respond to olfactory stimuli than females. Understanding factors that may lead to avoidance of virulent *M. anisopliae* strains by *C. formicarius* will be an essential part of developing an 'attract-and-infect' strategy for the management of *C. formicarius*.

1. Introduction

The sweetpotato weevil (SPW), *Cylas formicarius*, is an important pest of sweetpotato (*Ipomoea batatas* L.) in Papua New Guinea (PNG) (Sar, 2006). The adult female oviposits single eggs into tunnels that have been excavated in the roots or vine crowns and blocks the tunnels with a fecal plug (Sutherland, 1986a). Upon hatching, the larvae feed and tunnel into the root, and remain within the root for 20–30 days before pupation. The production of furanoterpenoids from larval tunnels leads to bad odours, making the roots unsuitable for human consumption (Ray and Ravi, 2005). Since sweetpotato is cultivated year-round in PNG, suitable host plants are always available for SPW. High levels of root damage are particularly evident in dry periods, when cracks in the soil expose the roots (Sutherland, 1986b). In PNG, most sweetpotato is grown in the highlands, with large quantities transported to the lowlands for sale at markets. Growers employ cultural methods to minimise SPW infestations, including selection of deep-rooted varieties, use of weevil-free planting material, and crop rotations. Growers have few other options for managing the pest since much of its life cycle occurs within the plant tissues, making chemical control particularly problematic (Bourke and Ramakrishna, 2009; Bourke and Vlassak, 2004; Talekar, 1991). This has led to a search for additional, ecologically friendly management procedures to contain the pest below threshold levels, and the development of entomopathogens for the

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control of SPW is seen as an area with particular potential.

The entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin has been used successfully on soil-inhabiting insects such as SPW, its congener *C. puncticollis* (Dotaona et al., 2015; Ondiaka et al., 2008; Rana and Villacarlos, 1991; Reddy et al., 2014b) and other coleopteran pests (Hajek et al., 2008; Nielsen et al., 2006). Some strains of this fungus have been developed as biological pesticides and registered to control various agricultural pests (de Faria and Wraight, 2007) although there are no *Metarhizium*-based biological pesticides registered in PNG. *Metarhizium* may be particularly useful for SPW control in PNG, where the high humidity may favour fungal survival and enhance infectivity. These factors have led us to conduct further work examining the behavioral responses of SPW to *M. anisopliae*.

Although insects are susceptible to entomopathogenic fungi when in contact with infective conidia (Rath et al., 1995), some arthropods have co-evolved a number of behaviors to limit infection (Roy et al., 2006 and references within). Grooming to achieve the removal of *Beauveria bassiana* conidia has been observed in the red imported fire ant *Solenopsis invicta* (Oi and Pereira, 1993; Siebeneicher et al., 1992). Alarm behaviors to deter other individuals from being exposed to nearby sources of *M. anisopliae* have been observed in termites (Rosengaus et al., 1999) and some insects may survive infection if they respond with strategies such as 'behavioral fever' (Blanford et al., 1998; Olesen, 1984). Behavioral strategies such as detection and avoidance have also been well documented and are of particular relevance to the current study. Both female and male *Coccinella septempunctata* (Coleoptera: Coccinellidae) have been reported to avoid conidia of *B. bassiana* (Ormond et al., 2011). When exposed to leaf surfaces bearing *B. bassiana* conidia and mycosed cadavers of conspecifics, the ladybird beetles avoided contact with both. Villani et al. (1994) investigated *Popillia japonica* (Coleoptera: Scarabaeidae) on turf grass and observed that adults and larvae avoided sites with high concentrations of *M. anisopliae* mycelia. Introduction of *Agriotes obscurus* (Coleoptera: Elateridae) into *M. anisopliae*-contaminated soil led to the beetles displaying avoidance behavior (Kabaluk and Ericsson, 2007). Staples and Milner (2000) investigated the repellency of *M. anisopliae* to termites (*Coptotermes lacteus*) using an agar tube method where they examined tunnelling and mortality. They broadly characterised isolates into three groups; (1) highly virulent isolates that resulted in limited tunneling towards *M. anisopliae* (high repellency); (2) low virulence isolates that caused low repellency and (3) highly virulent isolates that resulted in limited evidence of repellency. Based on these characteristics, Staples and Milner (2000) suggested the use of the third group of isolates in baiting trials to control termites. More recently, Mburu et al. (2009) reported *Macrotermes michaelseni* (Isoptera: Termitidae) avoiding a virulent *M. anisopliae* isolate in Y-tube olfactometer assays, also finding a correlation between virulence and repellency as shown by Staples and Milner (2000). In contrast, other studies have reported a lack of avoidance response by wasp parasitoids and *Anopheles* mosquitoes when confronted with the presence of lethal fungal pathogens on infected hosts and surfaces (Georgiev et al., 2013; Lord, 2001; Mnyone et al., 2010).

In a previous laboratory study, an *M. anisopliae* isolate (QS155) was shown to cause high mortality of adult SPW (defined here as > 50% mortality after 5 days or a LC_{50} of 1.7×10^5 conidia/ml 20 days after inoculation) (Dotaona et al., 2015). However, personal observations by the authors suggest that SPW may display avoidance behavior when exposed to sweetpotato roots inoculated with dry conidia or conidial suspensions of *M. anisopliae*. In this study, we investigated the response of SPW to the presence of *M. anisopliae* on agar cultures (in the presence of sweetpotato roots), and when applied to sweetpotato roots and whole plants. Responses to two *M. anisopliae* isolates of high and low virulence were also evaluated. The results of this study further improve our understanding of how best to utilise *M. anisopliae* for SPW control under field conditions.

2. Materials and methods

2.1. Insects and plants

SPW were maintained in an Adaptis growth chamber (Conviron Asia-Pacific Pty Ltd, Melbourne, Vic.; 26 ± 4 °C, 60% R.H., 12:12 h photoperiod) and provided with roots of the orange-fleshed sweetpotato variety 'Beauregard Gold' as food and for oviposition. Newly emerged adults were sexed using antennal morphology (Sutherland, 1986a) and separated one day post-emergence. Only 3-week old unmated adults were used in this study. Insects were starved for 24 h prior to all experiments.

'Beauregard Gold' sweetpotato roots were purchased locally. Sweetpotato plants were raised by burying roots in an all-purpose potting mix (Hortico Pty Ltd, Sydney, NSW) until they reached the vegetative stage in the glasshouse (28 ± 3 °C, 50–60% R.H). Shoots from the roots (~10 cm in length) with 1–2 fully developed leaves were cut and placed in polyethylene trays (BCS Plastics Pty Ltd, Sydney, NSW) with potting mix. After 3 weeks, the roots were transplanted into modified 140 mm black polyethylene pots (Garden City Plastics Pty Ltd, Dandenong South, Vic) containing heat pasteurized (90 °C for 3 h) sandy loam (Loam and Stone Pty Ltd, Wagga Wagga, NSW). The top of the soil was 3–5 mm below the hole drilled for fitting the olfactometer tubing. Plants were grown through for an additional month before use.

2.2. Fungus and spore preparation

In this study, two isolates identified as *M. anisopliae* (Dotaona et al., 2015) were sourced from Queensland, Australia. In a previous study, isolate QS155 caused high mortality of adult SPW whilst isolate QS002-3 had only low virulence (Dotaona et al., 2015). These isolates were used here to determine specifically whether the behavioral response of SPW varies between low and high-virulence isolates, and cultures have been lodged in the New South Wales Department of Primary Industries Herbarium with the accession numbers DAR 82480 and DAR 82876 for QS155 and QS002-3 respectively. Conidia were cultured on Sabouraud dextrose agar with yeast extract (SDAY) in 90 mm ∅ Petri plates. Three-week old sporulating cultures were used in this study for producing plugs/cores and conidial suspensions. SDAY plates were flooded with 10 ml of aqueous 0.05% v/v Tween 80® (Sigma-Aldrich Pty Ltd, Sydney), scraped using a sterile spatula, and the suspensions were poured into two 100 ml sterile tubes. Conidial concentrations were determined using an improved Neubauer haemocytometer and adjusted by dilution to 3.5×10^8 conidia ml^{-1}. The conidial suspension from each isolate was poured into a 100 ml Nalgene® aerosol spray bottle (ThermoScientific Pty Ltd, Sydney, NSW), with an outlet volume of ~0.36 ± 0.05 ml/s (equates to *ca.* $1.1 \times 10^8 - 1.4 \times 10^8$ conidia/s). Three fresh SDAY plates were spread plated with 25 µl of each isolate and incubated in the dark to determine germination rates after 24 h. Conidial germination of isolates QS002-3 and QS155 were 92 ± 1.5% and 87 ± 1% respectively.

2.3. Two-choice olfactometer construction

The two-choice olfactometer was constructed out of two 140 mm ∅ black polyethylene pots. A 10 mm ∅ hole was drilled 15 mm from the top of each pot. Black masking tape (uniform to the pots) was used to connect and seal each end of a 110 mm length of 8 mm ∅ clear vinyl tubing to the holes in the pots. A hole was then cut at the midpoint of the tubing and a 20 mm section of tubing was inserted to form the stem of a *T*-junction. A 50 ml plastic centrifuge tube was then cut 10 mm from its tip to leave an opening 8 mm in ∅, fitted into the 20 mm tubing to act as a 'release tube', and capped with a perforated lid (Fig. 1). A Rena 301 aquarium pump (Rena Aquatic Supply, Charlotte, NC) was used to create a steady airflow for volatiles from the arenas into the *T*-shaped tubing. A 50 cm length of 7 mm diameter clear vinyl