Frozen section and electron microscopy studies of the infection of the red palm weevil, *Rhynchophorus ferrugineus* (coleoptera:curculionidae) by the entomopathogenic fungus *Metarhizium anisopliae*

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**Abstract**

This study determined the pathogenicity of *Metarhizium anisopliae* strain SD-3 against invasive red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier (coleoptera:curculionidae) larvae in Hainan Province, China. Inoculation of $1 \times 10^8$ conidia/mL caused 100 % mortality of *R. ferrugineus*, indicating that the conidia of strain SD-3 were highly virulent. The process of invasion mechanism was showed by scanning electron microscopy (SEM) and frozen section as follows. Once *R. ferrugineus* was infected by strain SD-3, *M. anisopliae* hyphae first invaded the cuticular and body cavity of *R. ferrugineus*. Secondly, well-developed muscles, fat, tracheaes and digestive tube tissues in the abdomen of *R. ferrugineus* were then decomposed and absorbed by *M. anisopliae* hyphae, leading to the total destruction of the larvae. Finally, *M. anisopliae* hyphae reproduced, resulting in a large number of conidia in the body of RPW. The SEM and frozen section are convenient tools to observe the mode of action of entomopathogenic fungi and to observe how *M. anisopliae* is able to colonize and infect the host.

**Keywords:** *Metarhizium anisopliae*, *Rhynchophorus ferrugineus*, Frozen section, Electron microscopy studies

**Background**

Invasive red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier (coleoptera:curculionidae), is an important pests of a world range of palms of economic importance (Faleiro 2006). *R. ferrugineus* was originally reported in India, while now has been widely distributed in Asia, Africa, Australian (Fiaboe 2012; Kehat 1999; Mankin 2009). In China, *R. ferrugineus* is considered as a quarantine pest, and it has been found in 19 species of 15 palm genera (Dembilio et al. 2009). According to international standards for pest measurements (ISPM). This indicates *R. ferrugineus* can easily settle down in China, where it potentially poses a great threat to palm trees (Wu et al. 2007).

According to IPM strategy, several control methods have been used to *R. ferrugineus* invasion of palms. These methods include, cutting down and burning infected palms, trapping adult *R. ferrugineus*, chemical control, host plant resistance, bacteria control, virus control, nematodes mites control, parasitoid and predator insects, male sterile techniques and so on (Faleiro 2006; Francardi et al. 2013). Beside these, a number of entomopathogenic fungi (*Metarhizium anisopliae*, *Beauveri bassiana*, *Aspergillus sp.*, *Trichothecium sp.*, *Penicillium sp.*, *Fusarium sp.*) isolated from naturally infected *R. ferrugineus*
as a biological control agent against this weevil (Ghazavi and Avand-Faghih 2002; Gindin et al. 2006; Dembilio et al. 2010; Güerri-Agulló et al. 2011; Francardi et al. 2013). *Metarhizium anisopliae* is one of the most commonly studied species of entomopathogenic fungi, it is environmentally-friendly and harmless to human. However, *M. anisopliae* was discovered in naturally infected *R. ferrugineus* in Egypt and this strain caused a high mortality rate for larval and adult stages only under laboratory conditions (Merghem 2011; Cito et al. 2014).

Despite investigations of infection patterns and histopathology of *M. anisopliae* in selected insects is of economic importance, less study has documented the histopathology of *M. anisopliae* in *R. ferrugineus* (Toledo et al. 2010). Moreover, as the *R. ferrugineus* is highly promiscuous and adults live in aggregation, the fungi could spread in the population, infecting healthy insects by horizontal transmission, as suggested also by Llácer et al. (2013) and Francardi et al. (2013).

Scanning electron microscopy (SEM) has frequently been used to evaluate the infection process of entomopathogenic, Güerri-Agulló et al. (2010) used SEM to study the infection process of *B. bassiana* in *R. ferrugineus* cuticle and whole insects fungi versus their insect hosts. SEM studies by Alcides et al. (2002) provided a valuable insight into the mode of pathogenesis of *M. anisopliae* on western flower thrips. SEM studies of host–pathogen interactions have helped in determining some of the attributes of virulent fungal strains and in the identification of insect barriers to infection (Vestergaard et al. 1999). Frozen section has commonly been used in medical sciences for human pathological analysis due to its fast and stable identification effect (Gal and Cagle 2005). The method used in the preparation of SEM samples should avoid damage to the insect and fungal structures involved in penetration, especially when the objective is to document the infection process (Alcides et al. 2002). The purpose of the present studies, by using Electron Microscopy and frozen section methods, we examined the entire course of infection of *R. ferrugineus* by *M. anisopliae* with particular reference to histopathology of entomopathogenic fungi in *R. ferrugineus*, which could improve the effect of biocontrol and further expand its application in the field.

**Methods**

**Fungal isolate and preparation of suspensions**

Naturally-dead *R. ferrugineus* cadavers were collected from Wenchang, Hainan Island, China. Samples were soaked in 70 % alcohol for 1 min, and rinsed using sterile distilled water. The cadavers were subsequently surface-sterilized using 0.1 % mercury chloride, followed by three-time rinses in sterile distilled water. Part tissues were cut and inoculated on SDAY containing 40 g/L dextrose, 10 g/L peptone, 10 g/L yeast, 20 g/L agar and 500 μg/mL streptomycin. These tissues were separately placed on sterile petri dishes sealed with preservative film at 28 ± 1 °C, 75 ± 5 % RH for 6 days. Purification was achieved using a monospore culture, named SD-3. Scanning Electron Microscopy (SEM) was performed to study morphologic characteristics of SD-3 (Hitachi S-3000 N) (Driver et al. 2000; Su 2006).

**Experimental insects**

A laboratory population of *R. ferrugineus* was established by collecting larvae from infected palm trees in the Wenchang suburb, Hainan, China (Li et al. 2010). Larvae were reared on sugarcane stem tissues at 28 ± 1 °C, 75 ± 5 % RH. After adults emerged, they were placed in jars and supplied with cotton wicks saturated with 8–10 % honey for feeding. Subsequently, eggs were transferred to a moist sterile filter paper within an unsealed Petri dish (12 cm in diameter). Upon hatching, neonate larvae were individually transferred to 50 ml vials containing 10 g weevil’s artificial diet (Martin and Cabello 2006). About 7 days later, laboratory-reared larvae were obtained for further analysis.

**Laboratory bioassays**

We selected *R. ferrugineus* larvae of similar size during the feeding period. Conidia of divided purified SD-3 were placed in a sterile 10 mL centrifuge tube containing aqueous 0.1 % Tween 80 and vortexed the mixture for homogenization. Conidial concentration was determined using a hemocytometer. Dilution series of aqueous conidial suspension (1.0 × 10⁶) was prepared after proper modulation, then spayed on larvae. Treated larvae were separately transferred to 50 mL vials containing 10 g weevil’s artificial diet under controlled conditions (28 ± 1 °C, 80 ± 5 % R.H). Triplicate was performed for each trial with total 30 insects per process. After the treatment, insects were scored as dead at 24 h interval for 10 days. Meanwhile, dead larvae were selected and tested for potential histopathological changes (Tjandra and Melanie 2011).

**Symptom and histopathological changes of *R. ferrugineus* infected by *M. anisopliae***

Fresh larvae and dead adult were collected in laboratory bioassays avoiding water. Samples were dried via freeze-drying, coated with a gold palladium film, and observed using a Hitachi S-3000 N SEM. The frozen section was carried out on a tissue <1.5 cm wide and 3–5 mm thick. Several drops of optimum-cutting-temperature compound (OCT) were immediately added to the embedding medium on the chuck. The OCT bottom rapidly
showed in Fig. 1. The mycelium was smooth and
hyphal structure. R. ferrugineus cadavers were
penetrated, until appressoria were developed to start the
penetration stage at third day (Fig. 1f). Hydrophobic
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Results
Fungal morphology of fungi
The morphology of strain SD-3 was typical to the genus M. anisopliae. It grew fast on SDAY and PDA plates. Round colonies on agar plates were white or cream, with villous and the divergence of hollow hyphae on the surface. Punctiform olive green formed in the middle of the colony, which turned darker during growth and resulted in tawny color at the back of plates.

The morphology of hyphae and conidial of M. anisopliae SD-3 from R. ferrugineus cadavers were shown in Fig. 1. The mycelium was smooth and hyaline, well-branched and separated, with major hyphae up to 2.0–3.2 μm wide. Conidiophores were podgy, simple or highly branched, with 2–5 sterigma at the branch top. Conidia were colorless, elliptic and cylindrical, with obtuse ends. Most conidia were 4.8–5.5 μm × 2.0–2.5 μm in length. Growth temperatures were 28 °C.

Laboratory bioassays
The mortality rate positively increased with conidial concentration (P < 0.05). Conidia were found highly virulent to R. ferrugineus and caused approximately 100 % mortality 8 days post-inoculation of 1 × 10^8 Conidia/mL conidia. To the contrary, control group showed significantly lower mortality rates (3.33 ± 2.87 %)

Symptom and histopathological changes
SEM micrographs for cuticle and appendages of adult females were shown in Fig. 1b–g. Entomopathogenic fungi predominantly invaded R. ferrugineus through the external cuticle (Fig. 1a, e). When R. ferrugineus was treated with M. anisopliae SD-3, fungal propagules adhered to the cuticular surface and fungal conidia germinated, until appressoria were developed to start the penetration stage at third day (Fig. 1f). Hydrophobic conidia of SD-3 strain were found attached to all of the adult body, with a preference to surfaces containing hairs (Fig. 1c–e). SD-3 conidia were concentrated nearby and enclosed within the pores (Fig. 1g).

Results of light microscopy for larvae healthy and pathologic tissue were shown in Fig. 2a–j. Cuticle-degrading enzymes such as proteases, chitinases, and collagenases dissolved the cuticle, thus hyphae grew and branched while invading the integument and body cavity of the R. ferrugineus (Fig. 2b). M. anisopliae SD-3 conidia were found trapped and tightly bound to these hairs (Fig. 2h). Hyphae secreted different metabolic enzymes and destruXins. Well-developed muscles, fat and tracheae tissue (Fig. 2c, e, g) in the R. ferrugineus abdomen were decomposed and absorbed by hyphae, further formed the net structure (Fig. 2d, f, h). After entering the body cavity, hyphae seriously destroyed hemolymph, various tissues and pipelines. Digestive tube (midgut) was decomposed and absorbed by hyphae (Fig. 2i). Finally, hyphae reproduced and resulted in a large number of conidia in the R. ferrugineus body. Thus, the whole inner structure of R. ferrugineus was destroyed by M. anisopliae SD-3 hyphae.

Discussion
This study showed entomopathogenic fungus M. anisopliae SD-3 was highly virulent to R. ferrugineus, a serious pest of various palm species. Under the premise of harmless effect to environment or non-target organisms, biological control with pathogenic fungi would offer long-term insect control (Khetan 2001). The pathogenic fungi seem like to survival and distribute with the R. ferrugineus in its dark and humid surroundings. M. anisopliae is applied as conidia or mycelia in various formulations. By way of making the insects infected through the induction of a fungal epizootic, new conidia and viable cells are produced to spread to the health insects and thus achieve the control effect (Genthner et al. 1997). After the series of adhesion, prepenetration growth, penetration into the host, and settling down of the pathogen in the host, the insects would be infected by M. anisopliae (Lattanzio et al. 2006).

Most entomopathogenic fungi enter in the host by penetrating through the host cuticle. In the course of fungal infection, the fungi are adsorbed on the host cuticle in the first step before penetration (Urquiza and Keyhani 2013). Dong et al. (2009) proposed adhesion to occur at three successive stages: (1) adsorption of the fungi propagules to the cuticular surface; (2) adhesion or consolidation of the interface between pre-germinant propagules and the epicuticle; (3) fungi germination and development at the insect cuticular surface, until appressoria are developed to start the penetration stage. Infection will proceed after a successful penetration being achieved.
addition, we found intersegmental membrane and hair was the first spot invaded by *M. anisopliae* when *R. ferrugineus* larvae were infected by this entomopathogenic fungus (Fig. 1). This could be attributed to relatively thin chitin layers in the intersegmental membrane, which favored infection by germ tubes. During the cultivation of dead larvae on moist sterile filter paper, white hypha first occurred at the intersegmental membrane and hair associated with most abundant conidia, as well as longest germ tubes and hyphae. In addition, germination of conidia was first observed in the intersegmental membrane. Together these observations indicated that intersegmental membrane and hair was the weak point subject to *M. anisopliae* infection.

Histological section showed that before *R. ferrugineus* larvae died due to infection, associated body tissues were affected to different degrees, leading to obvious lesions. Direct death reason of the host remained unclear. It was likely that larva body tissues were seriously damaged due to infection by *M. anisopliae*, preventing normal larva physiological activities. Along with mycelia growth, larva bodies (muscles, fat, tracheae digestive tube) were occupied by a large amount of fungal hypha (Fig. 2), which exhausted nutrients and impeded fluid circulation, leading to physical starvation and metabolic disorders. Thus, hypha invasion could cause body tissue failure of larva, which died due to the incapability of normal physiological activities.

**Fig. 1** Scanning electron microscope micrographs of *Metarhizium anisopliae* SD-3 on *R. ferrugineus*. a *M. anisopliae* SD-3 hyphae (arrows) enclosed with the hair situated on the cuticle; b *M. anisopliae* conidia in the second antennal segment (arrows); c *M. anisopliae* conidia and hyphae in the antennal sensory hairs; d *M. anisopliae* conidia and hyphae (arrows) grow on the hairs of the abdomen; e *M. anisopliae* germ tube penetrating through the cuticle of the abdomen with the adult female; f *M. anisopliae* conidia and hyphae (arrows) enclosed within the cuticle and hairs of the tibia; and g *M. anisopliae* conidia (arrows) near to the spiracle.
Another reason caused larva death might be physiological and biochemical changes in infected larva body. Sloman and Reynolds (1993) suggested mycotoxin as the true reason for the death of insect infected by many imperfect fungi. Such toxin not only inhibits the immune function of host, but also affects associated central nervous system and has partial pathogenic effect, thus promoting the death of host. However, Wang and You (1999) indicated the uncertainty in functional mechanism of such toxin during relevant infection process. These authors only found pure toxin caused immune pressure, muscle paralysis and malpighian tubule damage in the host. Further study is needed to demonstrate whether mycotoxin produced by *M. anisopliae* was the primary reason caused the death of *R. ferrugineus*.

We used SEM and frozen section to observe the fungal infection. We also first applied frozen section to study histopathological mechanisms of *M. anisopliae* SD-3, and developed a set of methods suitable for studying high-fat larvae. In contrast, research of insect pathology has mainly employed traditional paraffin section, which usually takes 3–5 days for sample preparation and pre-treatment. By comparison, frozen section only requires 30–60 min for sample processes. This contributed to experiment effectiveness and reduced impacts of prolonged fixation on samples.

When collecting fresh samples of histological sections, caution must be taken to avoid water. Temperature is the most important factor for frozen section. As larvae contain substantial fat, high temperature may cause adhesive due to incomplete freeze. On the other side, low temperature may increase the fragility of samples which are easy to break during sectioning. Based on our observations in this work, we suggest rapid freeze at $-28 \pm 1 \, ^\circ C$ and section at $-22 \pm 1 \, ^\circ C$ yield optimum results.
Conclusions
In summary, this study showed high susceptibility of *R. ferrugineus* larvae to certain concentrations of local *M. anisopliae* SD-3. We further demonstrated the histopathological mechanism of this entomopathogenic fungus in *R. ferrugineus*. This study allowed the observation of the different phases of the disease cycle, and further demonstrated the importance of understanding these phases in selecting isolates for biological control of *R. ferrugineus*.

Abbreviations
*M. anisopliae*; *M. anisopliae* var. *Dcjhyium*; RPW; *R. ferrugineus*; *Rhynchophorus ferrugineus* Olivier (coleoptera: curculionidae).

Authors’ contributions
XS and WQ conceived this study. WY, JZ and XN collected the reference data, FL corrected the manuscript. All authors designed and evaluated the experiments. XS mainly and GM partly performed the experiments. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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