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Histopathological and molecular insights into the ovicidal activities of two entomopathogenic fungi against two-spotted spider mite

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ABSTRACT

Entomopathogenic fungi can infect and kill spider mite eggs but their ovicidal activities are poorly understood. Here we gain histopathogenical and molecular insights into the ovicidal activities of *Beauveria bassiana* and *Isaria fumosorosea* against the two-spotted spider mite, *Tetranychus urticae*. Scanning electronic microscopy indicated successful adhesion and germination of fungal conidia on egg shell at 24 h post-spray (HPS). Germ tubes of both fungi could penetrate into egg shell with penetration pegs at 48 HPS. Interestingly, the germ tubes of *B. bassiana* may elongate on egg surface to locate appropriate sites for penetration, acting as 'searching' hyphae. Aside from the normal penetration, the germ tubes of *I. fumosorosea* can be completely or partially embedded into egg shell for a distance of extension, forming shell humps. Light microscopy of ultrathin sections of infected eggs showed shrunken (affected) or disrupted embryos at 48–96 HPS despite little effect on egg cleavage at 24 HPS. However, distinguishable hyphal cells were hardly found inside the embryos lacking oxygen although fungal outgrowths were abundant on unhatched (killed) eggs. In PCR with specific probes, the 18S rDNA signals of *B. bassiana* (412 bp) and *I. fumosorosea* (454 bp) in the DNA extracts from surface-cleaned mite eggs increased at 0–96 HPS, confirming fungal colonization in the infected eggs. We consider that the colonization on shell surface and underside could rely upon extending hyphae for uptake of egg nutrition, resulting in embryo disruption. Our observations add knowledge to microbial control of spider mites.

1. Introduction

Spider mites (Acari: Tetranychidae) are phytophagous mites that usually live on the undersides of plant leaves and cause severe damage by puncturing plant cells. For instance, the two-spotted spider mite, *Tetranychus urticae* Koch with the synonym of *T. cinnabarinus* (Boisduval) (Ros and Breeuwer, 2007), can infest hundreds of plants, including vegetables, food crops and ornamentals (Hazan et al., 1975). Nymphs hatched from small-size eggs develop into adults, ~500 μm in length, within 10–12 days at 25 °C or 6–7 days at 30 °C (Hazan et al., 1974). On average, each female adult may lay ~160 eggs at 25 °C or 60–80 eggs at 30 °C (Hazan et al., 1974; Bhagat and Singh, 1999).

Egg quantity and viability are crucial for the development of spider mite populations (Gotoh et al., 2006; Bostanian et al., 2007) and hence the targets of the population control. For a long run, spider mite control has largely relied upon chemical acaricides, of which only a very few are ovicidal (Singh et al., 1975; Guo et al., 1998; Dagli and Tunc, 2001). However, spider mites have developed high resistance or cross-resistance to almost all chemical acaricides and insecticides (Grosscurt et al., 1994; Campos and Omoto, 2002, 2006; Yamamoto et al., 1996), making it an urgent need to develop an alternative control strategy that is based on the use of fungal biocontrol agents against phytophagous mites (Chandler et al., 2000; van der Geest et al., 2000).

In the past decade, entomopathogenic fungi, such as *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea*, have been found causing high mortalities of several phytophagous mites under laboratory conditions, including *T. urticae* (Alves et al., 2002; Shi et al., 2008a; Bugeme et al., 2009), *T. evansi* Baker and Pritchard (Wekesa et al., 2005, 2006), and *Ployphagotarsonemus latus* (Banks) (Pena et al., 1996; Maketon et al., 2008). Aside from promising virulence to immature and adults, some fungal strains showed ovicidal activities to spider mites (Shi and Feng, 2004; Wekesa et al., 2006). The ovicidal activity of a *B. bassiana* strain was greatly enhanced by the application of sublethal pyridaben rates (Shi et al., 2005). An oil-based, emulsifiable formulation of the same strain

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can kill 50% of *T. urticae* eggs at the spray rates of 65–320 conidia/mm^2 under the conditions of 20–30 °C and 51–95% relative humidity (Shi et al., 2008b). For these reasons, the application of fungal formulations may provide desirable controls of citrus red mite *Panonychus citri* (McGregor) in orchards (Shi and Feng, 2006) and spider mite complex in cotton fields (Shi et al., 2008c).

Spider mite eggs lack hemocoel, in which fungal cells can propagate by budding after penetration into the cuticles of active immature and adults, and are well protected by their shells, a barrier to external hazards threatening the developing embryos (Witalinski, 1993). Egg cleavage, the first stage of embryonic development, usually begins ca. 4 h after oviposition, followed by the temperature-dependent progression of segmentation and internal organization (Mariela et al., 1992). Although fungal sprays may kill high proportions of spider mite eggs in conventional bioassays (Shi and Feng, 2004; Wekesa et al., 2006), little is known about the progression of fungal infection to mite eggs and subsequent histopathological effect on embryonic development. Such information is important for understanding the ovicidal activities of fungal biocontrol agents because terrestrial arthropod eggs have 'solid' egg shells that are largely composed of crystal proteins and have very limited aeropyle openings for breathing (Hinton, 1969). Therefore, this study sought to gain an insight into the ovicidal activities of *B. bassiana* and *I. fumosorosea* against *T. urticae* via histopathological and molecular examination of sprayed mite eggs.

2. Materials and methods

2.1. Fungal isolates and cultures

The strain *B. bassiana* ARSEF 2860 (Bb2860 herin) was originally isolated from a naturally mycozed aphid cadaver (Feng et al., 1990). Pfr116, an isolate of *I. fumosorosea* (previously *Pacelomyces fumosoroseus*) from *Bemisia* sp., was provided by T.J. Poprawski (USDA-ARS, Biological Control of Pests Research Unit, Weslaco, TX). The two isolates were selected for this study due to their high virulence to *T. urticae* eggs (Shi and Feng, 2004) and females (Shi et al., 2008a). Both isolates were stored on slants at −72 °C and recovered on Sabouraud dextrose agar plus 1% yeast extract (SDAY) at 25 °C for ~7 days, followed by production of aerial conidia as follows.

2.2. Preparation of aerial conidia and mite eggs

Aerial conidia of Bb2860 and Pfr116 were produced on steamed rice (Ye et al., 2006), harvested through a vibrating sieve and dried to a water content of ~5% at ambient temperature on a vacuum drier following a previous protocol (Shi et al., 2008b). The dried conidia were sealed in glass tubes and stored at 4 °C for use as soon as possible. Conidial viability exceeded 92% at the time of use.

A laboratory stock of *T. urticae* was maintained on caged fava bean (*Vicia faba* L.) plants in a walk-in growth room at the regime of 23 ± 2 °C and 12:12 L:D. To obtain *T. urticae* eggs at uniform age, 20 adult females were arbitrarily taken from the plants and transferred onto a detached leaf with hairy roots growing from its petiole into 1.5% agar in Petri dish (9 cm diameter) and allowed to freely lay eggs for 12–18 h. The females were then removed, leaving 30–45 eggs per leaf for use.

2.3. Fungal spray

For each strain, a suspension of 1×10^8 conidia/ml 0.02% Tween 80 was prepared. More than 150 batches of 30–45 eggs on the bean leaves in uncovered Petri dishes were separately exposed to a spray of 2 ml conidial suspension (treatment) or of the same volume of 0.02% Tween 80 (blank control) from the top nozzle of Automatic Potter Spray Tower (Burkhard Scientific Ltd., Uxbridge, Middx, UK) at the uniform working pressure of 0.7 kg/cm^2. After exposure, all batches of sprayed eggs on the leaves in Petri dishes were kept at room temperature for 5 min, covered with lids and then maintained for up to 6 days at 25 ± 1 °C and 12:12 L:D. During the period, 30–35 batches of eggs (adjusted in terms of egg counts on leaves) were collected from the treatment and the control, respectively, at 12 or 24 h interval to warrant the following analyses.

2.4. Scanning electronic microscopy (SEM)

To observe the infection progression of sprayed conidia on egg surface, spider mite eggs collected at earlier and last schedules were carefully transferred to one side of double-sided tape with another side attaching to a piece of cellophane. Three or four pieces of cellophane with each vectoring ~10 eggs were fixed overnight at 4 °C in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), followed by rinsing three times (10 min each) with 0.1 M phosphate buffer (pH 7.2). The fixed eggs were dehydrated in the gradients of 30–100% ethanol (5 min each), further dried to critical point in CO_2 (CPD 030 BALTEC), and coated with gold in a sputter-coater (SCD 050 BALTEC). All the eggs were then examined via SEM.

2.5. Histopathological examination

Fifty infected or uninfected eggs from daily collection were embedded into 2% agar plug (10 mm diameter and 3 mm thick). All the agar plugs were immersed in buffered Bouins fluid (volume ratios of acetic acid, formalin and picric acid: 1:5:15) for 12–24 h, dehydrated in the gradients of 70–100% ethanol, and then embedded in paraffin. Ultrathin sections (5 μm) of mite eggs were stained with hematoxylin–eosin, followed by microscopic examination to observe possible histopathological changes inside infected eggs versus uninfected ones.

2.6. PCR detection of fungal genomic DNA in infected eggs

To extract total DNA of each target fungus from infected mite eggs, ~1000 eggs from the daily collection (treatment or control) were suspended in 100 μl PBS buffer (135 mM NaCl, 2 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM KH_2PO_4, pH 7.4) in 1.5 ml centrifuge tubes, followed by adding 1 ml of 0.02% Tween 80 to the tube. The egg suspension was thoroughly vortexed and centrifuged for 6 min at 500*g*. The whole washing process was repeated three times to remove contaminated materials (including sprayed conidia and their germlings) on egg surface. Maintained for 1 h at −20 °C, the washed eggs were ground in liquid nitrogen. The homogenized mixture was suspended into 600 μl of DNA extraction solution (100 mM Tris–HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA, 1% SDS, 0.15 mM spermine, and 0.15 mM spermidine). Subsequently, 10 μl of 10% SDS, 50 μl of RNase (20 mg/ml) and 5 μl of proteinase K were added to the suspension, followed by 1 min vortex and 2 h incubation at 37 °C. DNA extracts were obtained using the methods of phenol–chloroform extraction and ethanol precipitation (Zou et al., 2006), resolved in 20 μl dd-H_2O and stored at −80 °C for use.

To confirm fungal colonization inside the eggs, the 18S rDNA sequences of *B. bassiana* (~410 bp) and *I. fumosorosea* (~450 bp) were amplified from Bb2860 and Pfr116 via PCR with the respective forward primers ACCTCAAACTCCTGTATTCCAGCA and CCTCGCGCCGG CCCGCGA, and the same reverse primer CAGCGG GTAGTCCTACCTGA TCCGAGG. The PCR system (20 μl) consisted of 1 μl of *Taq* polymerase (Takara™ DNA Polymerase, Takara, Japan), 2 μl of 10 × PCR buffer, 2 μl of $MgCl_2$ (25 mM), 0.5 μl of deoxynucleotide triphosphates (10 mM), 0.5 μl of each primer (10 μM), 13.5 μl of dd-H_2O and 0.5 μl