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Evaluation of *Metarhizium anisopliae* (Deuteromycota: Hyphomycetes) for control of broad mite *Polyphagotarsonemus latus* (Acari: Tarsonemidae) in mulberry

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Abstract A study on 12 entomopathogenic fungi for controlling broad mite (*Polyphagotarsonemus latus* (Banks)) in mulberry found that *Metarhizium anisopliae* CKM-048 was the most virulent strain in controlling both larvae and adult broad mites at the concentration of 2×10^8 conidia/ml. There was no ovicidal effect when tested with broad mite eggs. Median lethal concentrations (LC_{50}) of *M. anisopliae* in killing larvae and adults were 8.7×10^6 and 1.3×10^7 conidia/ml, respectively. Median lethal times (LT_{50}) of larvae and adults were 2.4 and 3.8 days, respectively, at the concentration of 2×10^8 conidia/ml. The fungus was found to produce protease and chitinase. Scanning electron microscope (SEM) studies were done to monitor the infection steps of the fungus on broad mites. A greenhouse test on mulberry trees revealed that *M. anisopliae* could reduce the broad mite population within 4 days after treatment. However, after 7 days, its efficacy was decreased significantly.

Keywords Broad mite · *Polyphagotarsonemus latus* · *Metarhizium anisopliae* · Entomopathogenic fungi · Mulberry

Introduction

Broad mite (*Polyphagotarsonemus latus* (Banks)) has a world-wide distribution and is known by a number of common names. In Thailand it is called the yellow mite, and it is a serious problem in areas where chili (*Capsicum annum*) is cultivated (Kemsawasd 1976). Besides chili, a wide variety of agricultural crops, ornamentals, and wild plants have been recorded as hosts (Jeppson et al. 1975; Yang and Chen 1982; Ibrahim and Low 1998; Pena

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and Campbell 2005). In the Philippines, this mite is a pest of young plants including tomato, potato, tobacco, and ornamental plants.

White mulberry (*Morus alba*) is a short-lived, fast-growing, about 15–20 m tall tree. It is native to eastern Asia and its natural fruit color is deep purple. The leaves are the preferred feedstock for silkworms (*Bombyx mori*). White mulberry is extensively planted throughout the warm temperate Northern Hemisphere, mainly for the silk industry. Recently tea made of mulberry leave has become popular in some countries. Besides thrips and whitefly, broad mite is one of the serious sucking pests on mulberry leaf. Some chemical pesticides are being used, such as sulfur and amitraz, but they caused some phytotoxic effect, especially during the summer season, with ambient temperature as high as 40°C. Therefore, an integrated pest management program has initiated, employing predators and parasites, but also microbial miticides. Nugroho and Ibrahim (2004) have reported on a laboratory bioassay of three entomopathogenic fungi against broad mites on chili. They found that the most virulent strain was *Paecilomyces fumosoroseus* followed by *Beauveria bassiana* and *Metarhizium anisopliae*.

In this study, several entomopathogenic fungi were tested for their ability to control broad mite adults, larvae, and egg stages on mulberry leaves in both laboratory and greenhouse conditions. The lethal concentration (LC_{50}) and time (LT_{50}) were evaluated for the most virulent fungus. Enzyme production from the fungus was studied. Microscope and scanning electron microscope (SEM) studies for the fungal infection steps were performed.

Materials and methods

Preparation of conidial suspension

Eighty-eight soil samples and 75 homopteran, 220 isopteran, and 86 hemipteran cadavers were sampled from northeastern and central parts of Thailand. Twelve entomopathogenic fungi were isolated by the method of Meikle et al. (2005) and sent to the Thailand Institute of Scientific and Technological Research (TISTR, Bangkok, Thailand) for identification.

All screen cultures were grown on potato dextrose agar (PDA) (Difco, Becton-Dickson, Sparks, MD, USA) at 27 ± 1°C in the dark for 14 days. Conidial suspensions were made by lightly scraping the fungal culture surface with a sterile cell spreader into a 100-ml plastic container. The conidial clumps were suspended in distilled water with 0.01% Tween 80 (ICI Americas, Norwich, NY, USA). The suspensions were vortexed for 5 min to dissociate clumps and then filtered through one layer of cheesecloth to remove conidial clumps and mycelial debris. Concentration of each suspension was diluted to 2×10^8 conidia/ml determined by a Neubauer hemocytometer under a phase-contrast microscope. The suspensions were used on the same day or the day after preparation and shaken before use. The pure fungal culture of the most virulent strain was deposited at TISTR.

Preparing mulberry trees for rearing broad mite

A mulberry stalk of 1 cm diameter and 30 cm length was transferred into a 20-cm diameter pot. The pots were placed in a cage (60 × 60 × 200 cm) and covered with a nylon sheet to prevent infestation with pests. For the laboratory and greenhouse tests, 20 and 40 pots were prepared, respectively. After new leaf growth emerged to about 10 leaves, 20 male and 20 female adult broad mites per pot were randomly introduced on the leave surface. Broad mites were obtained from the Thai Department of Agriculture, where they had been reared

on mulberry. Broad mites were transferred by a single-hair brush pen. All work was done under a stereo microscope.

Screening for the most virulent fungus against broad mite adults, larvae and eggs

Fourteen treatments consisting of 12 entomopathogenic fungi with an untreated control and a water treated control were performed in three replicates. A mulberry leaf without broad mites was cut into a 5-cm diameter circle and placed on moist cotton wool in an autoclaved 9-cm Petri dish. Twenty broad mite adults or larvae were transferred into each dish using a single-hair brush pen. For the assays with eggs, 20 adult female mites were placed on a clean mulberry leaf disc for 6 h at room temperature. Then females were removed and the number of eggs laid was reduced to 20 per leaf disc by puncturing the excess eggs with a needle.

Aliqouts of 1 ml of a fungal suspension were sprayed into a dish using a thin-layer chromatography (TLC) sprayer (Merck, Germany), resulting in $\sim 1 \times 10^7$ conidia/cm^2 leaf surface (Cuthbertson and Walters 2002). Observations were made every 12 h to check for broad mites falling off the leaf margin. Every day for 5 days broad mite adult and larva mortality (%) was recorded, or the percentage of unhatched eggs. All observations were done under a stereomicroscope.

For confirmation of fungus infestation of the dead broad mites, cadavers were dipped in a 10% sodium hypochlorite (NaOCl) solution (Sigma-Aldrich, MO, USA) for 5 min, allowed to dry, placed on PDA, and monitored for possible mycelium germination.

Median lethal concentration (LC_{50}) and time (LT_{50}) assessment

The most virulent fungus was further studied for its median lethal concentration (LC_{50}). Suspensions were prepared at 2×10^6, 2×10^7 and 2×10^8 conidia/ml and tested on larvae and adults separately (seven replications per treatment). Mortality was checked daily for 3 days. For the median lethal time (LT_{50}), a suspension was prepared at 2×10^8 conidia/ml and tested on larvae and adults. Mortality (%) of broad mites was recorded daily for 5 days.

Analysis of enzymes produced by the most virulent fungus

Qualitative study

The most virulent fungus was cultured on PDA for 14 days at room temperature (27 ± 1°C) in the dark. A 1-cm diameter piece of agar covered with mycelium was cut using a cork borer, placed on skimmed milk agar (Difco, Becton-Dickson, Sparks, MD, USA) and incubated in the dark at room temperature for 7 days. The occurrence of clear zones depicts protein-digesting enzymes produced by the fungus. The efficacies of enzyme production can be determined in terms of the ratio between the clear zone diameter and the colony's diameter (Chongcharoen and Vatanyoopaisan 2005). To investigate chitin-digesting enzymes, the fungus was cultured on chitin agar (chitin from crab shells from Sigma-Aldrich, MO, USA) (Chatdumrong 1996).

Quantitative study

Methods of Khan et al. (2003) and Dackman et al. (1989) were employed. For protease production, 0.2% (W/V) skimmed milk (Sigma-Aldrich) was added to minimal-medium

(Bonants et al. 1995) cultures in 1-l conical flasks containing 250 ml culture medium and inoculated with the most virulent fungus at 1.0×10^6 conidia/ml. Cultures were incubated on a shaker at 125 rpm for 7 days at 27°C. Samples were drawn daily and diluted in a carbonate-bicarbonate buffer pH 10.2 (Dawson et al. 1969). One milliliter of diluted sample was incubated with 1 ml of 1% azocasein (Sigma-Aldrich) at 50°C for 30 min. After that, the reaction was terminated by the addition of 1.5 ml 5% trichloroacetic acid (Sigma-Aldrich). Non-digested azocasein was separated by centrifuging at 600*g* for 20 min and culture supernatants filtered through a 0.2-μm membrane. Protease activity was measured at 345 nm and expressed as mg dry weight per ml azocasein solubilized, based on the formula $A_{345} \times 0.566 \times$ dilution factor (Lovrien et al. 1985). Protein content of the filtrate was determined by the method of Lowry et al. (1951).

For chitinase production, minimal medium was supplemented with 0.5% (W/V) chitin (Sigma-Aldrich). One-liter conical flasks containing 250 ml medium were inoculated with 1.0×10^6 conidia/ml of the most virulence fungus and incubated at 125 rpm for 7 days at 27°C. Daily, 1.5 ml of sample was drawn and incubated with 1 ml of 1% colloidal chitin at 37°C for 2 h (Elad et al. 1982). Precipitate was separated and the supernatant was measured at 285 nm. Chitinase activity was indicated by the increase in absorbance of *N*-acetyl-L-glucosamine.

Infection characteristics of the most virulent fungus against broad mite larvae

Twenty larvae were transferred onto a clean mulberry leaf lined with moist cotton wool and placed in a Petri dish ($n = 5$). A suspension of 2×10^8 conidia/ml was sprayed on the leaf. For the light (stereo) microscope study, cadavers were collected after 5 days, rinsed with distilled water and dipped into Nesbitt solution (40 g chloralhydrate crystals, 10 ml HCl, 40 ml water; Sigma-Aldrich), for 10 min until the body was cleared. Infection stages were photographed.

For the SEM study, the leaves filled with larvae were collected after 24, 48, 72, 96, and 120 h after the start of infection. Due to the tiny size of the broad mite, the leaf was cut into 1-cm^2 portions, placed into a micro well plate. The plate was kept in a 2.5% glutaraldehyde solution (Sigma-Aldrich) adjusted with 0.2 M cacodylate buffer (Sigma-Aldrich) to pH 7.2 for 12 h at 4°C. The sample was then rinsed three times with 0.1 M cacodylate buffer pH 7.2 for 10–15 min each. The rinsed sample was placed in 1% osmium tetroxide solution (Sigma-Aldrich) at pH 7.2 for 1 h at 4°C. The rinse with 0.1 M cacodylate buffer was repeated three more times and the final sample was washed with an increasing series of 30%, 50%, 70%, 90% and 100% acetone solutions (Merck, NY, USA). Using the critical point drying technique (Denton Vacuum, Cherry Hill, NJ, USA) with CO_2, the samples were dried and fumed with carbon and gold vapors, respectively (Wagner and Lewis 2000). The samples were observed under a SEM (Hitachi S-2500).

Greenhouse study of the most virulent fungus against broad mite

Forty broad mites on a mulberry leaf were prepared as described earlier. Two suspensions of 2×10^7 and 2×10^8 conidia/ml, resulting in $\sim 1 \times 10^6$ and 1×10^7 conidia/cm^2, respectively, were applied as described earlier. Amitraz 20% EC (Mitac™, AgrEvo) at 60 ml per 20 l water, resulting in ~0.01 mg a.i./cm^2, was applied for comparison, as were an untreated and a water-treated control. All five treatments were conducted in a completely randomized design ($n = 8$). Average temperature ranged between 26 and 34°C and relative humidity was $80 \pm 5\%$.

One day before treatment, juvenile plus adult broad mites were counted on five young leaves at the top of the 120-cm-tall plants. A TLC sprayer was used to treat the whole plant, especially upper and lower leaves. Broad mites were counted at 1, 4, and 7 days on leaves on the plants.

Statistical analysis

In the assays screening for virulence, treatment effects were corrected for control mortality using Abbott's (1925) formula. Data from each trial were analyzed using analysis of variance (ANOVA) and means were compared by Duncan's new multiple range test (DMRT). Toxicological data were analyzed by Probit analysis (Finney 1971) with 95% confidence intervals, using SAS version 9.1.3. The greenhouse data were analyzed by ANOVA, because no significant difference was observed before spraying.

Results

Screening for the most virulent fungus against broad mite adults, larvae and eggs

Twelve entomopathogenic fungi were clarified (Fig. 1). After 5 days at room temperature ($27 \pm 1°C$) and $72 \pm 2\%$ r.h., *M. anisopliae* CKM-048 yielded the highest mortality (mean $\pm$ SE, $71.7 \pm 6.0\%$) of broad mite adults. Larval mortality was highest by *M. anisopliae* CKM-048 (100%) and *B. bassiana* CKB-048 ($95 \pm 5\%$) (Fig. 2). The 10 remaining isolates caused significantly lower larval mortality than these two isolates. Dipping of the cadavers in NaOCl solution and incubating them on PDA, confirmed that all new mycelia emerged from the cadavers were related to the original isolates. None of the 12 isolates had ovicidal activity: eggs in all treatments hatched within 1–2 days, so no fungal isolate in this experiment was effective in controlling broad mite eggs.

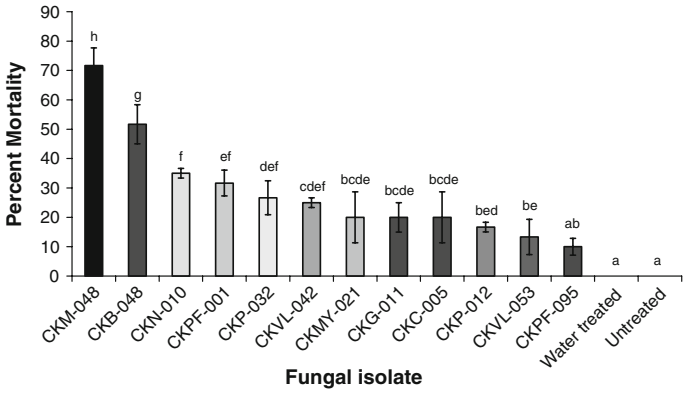

Fig. 1 Mortality (mean %) of broad mite adults on mulberry leaf discs, 5 days after being sprayed with suspensions from 12 entomopathogenic fungi. Bars with different letters are significantly different ($P < 0.05$, DMRT). The fungi are: *Beauveria bassiana* CKB-048, *Paecilomyces lilacinus* CKP-012, *P. lilacinus* CKP 032, *P. fumosoroseus* CKPF-001, *P. fumosoroseus* CKPF-095 (reclassified in part as *Isaria fumosorosea*; Luangsa-Ard et al. 2005), *Gliocladium virens* CKG-011, *Verticillium lecanii* CKVL-042, *V. lecanii* CKVL-053 (reclassified in part as *Lecanicillium muscarium* (Petch); Zare and Gams 2001), *Nomuraea rileyi* CKN-010, *Cordyceps brongniartii* CKC-005, and *Myrothecium verrucaria* CKMY-021

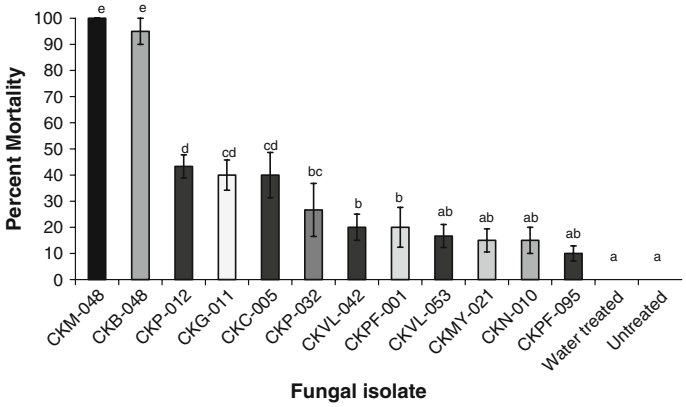

Fig. 2 Mortality (mean %) of broad mite larvae on mulberry leaf discs, 5 days after being sprayed with suspensions from 12 entomopathogenic fungi. Bars with different letters are significantly different ($P < 0.05$, DMRT). Fungi: see Fig. 1

Based on these results, *M. anisopliae* CKM-048 was coined the most virulent fungus and selected for further experiments.

Median lethal concentration (LC_{50}) and time (LT_{50})

The *M. anisopliae* CKM-048 suspension causing 50% mortality in broad mite larvae was found to lay between 2×10^6 and 2×10^7 conidia/ml. Therefore, two additional concentrations, 4×10^6 and 1×10^7, were prepared and tested. Probit analysis showed that the calculated LC_{50} for broad mite larvae was 8.71×10^6 conidia/ml. A similar procedure was done with adults, resulting in an LC_{50} of 1.32×10^7 conidia/ml (Fig. 3a).

At a concentration of 2×10^8 *M. anisopliae* CKM-048 conidia/ml, the LT_{50} for larvae was 2.36 days, and for adults 3.82 days (Fig. 3b).

Enzymes produced by the most virulent fungus

After 7 days incubation, *M. anisopliae* CKM-048 produced a protein- and a chitin-digesting enzyme. The ratio between clear zone and colony diameter that occurred on skimmed milk agar was 1.36 and on chitin agar it was 1.09. The quantitative study confirmed that the fungus was capable of producing protease and chitinase activity (Table 1).

Microscopic study of infection characteristics

After dipping a broad mite cadaver infected with *M. anisopliae* CKM-048 in Nesbitt solution, the cadaver became transparent and mycelium was seen inside and outside the body, whereas no mycelium was seen in the uninfected broad mite (Fig. 4).

A series of SEM pictures illustrates the infection process. At 1–2 h after contact, the conidia of *M. anisopliae* CKM-048 adhered to the cuticle of a broad mite larva (Fig. 5a). After 48–60 h, the fungus started to germinate and penetrate into the broad mite's body (Fig. 5b, c). During 72–96 h, fungal mycelia extrude from the broad mite and cover most of its body (Fig. 5d, e). Finally, after 120 h, the fungus started conidiogenesis and new conidia were formed (Fig. 5f).

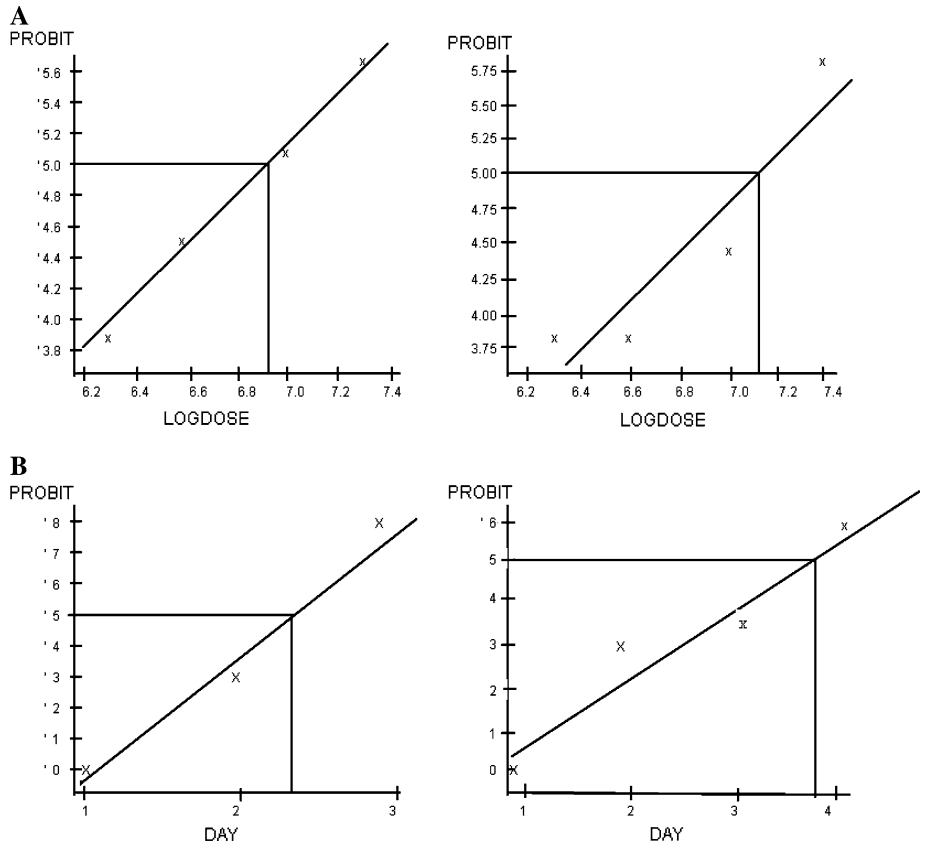

Fig. 3 Median lethal concentration (LC_{50}) of *Metarhizium anisopliae* CKM-048 suspensions and median lethal time (LT_{50}) when treated with *M. anisopliae* CKM-048 at 2×10^8 conidia/ml. (**A**) LC_{50}, and (**B**) LT_{50}, of broad mite larvae (left) and broad mite adults (right)

Table 1 Enzyme production by *Metarhizium anisopliae* CKM-048 on agar

Day	Protein (mg/ml)	Protease activity	*N*-acetyl-L-glucosamine	Chitinase activity
1	0.911	0.0000	0.122	0.0819
2	0.567	0.0137	0.143	0.0837
3	0.472	0.0176	0.222	0.0902
4	0.480	0.0279	0.222	0.0938
5	0.588	0.0245	0.268	0.0992
6	0.574	0.0339	0.296	0.1016
7	0.567	0.0475	0.323	0.1056
Control	0.790	0.0000	0.163	0.0000

Greenhouse study of the most virulent fungus against broad mite

Before application, the average number of broad mites found in all treatments did not differ significantly, hence the data could be analyzed by ANOVA. One day after spraying, amit-

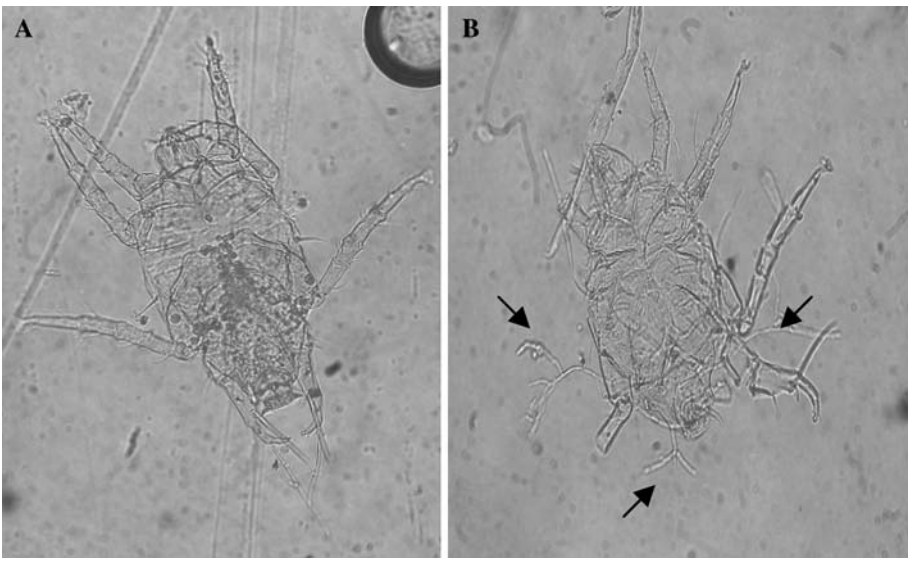

Fig. 4 Broad mite cadavers under the light (stereo) microscope: (**A**) Uninfected mite, no fungal mycelium inside the cadaver (control). (**B**) Mycelium of *Metarhizium anisopliae* CKM-048 extruded out of the infected cadaver (indicated by *arrows*)

raz had immobilized almost all broad mites, whereas the numbers of moving broad mites in the other four treatments were all about the same as before treatment (Table 2).

After 4 days, amitraz had killed all mites. The highest concentration of *M. anisopliae* CKM-048 suspension had killed significantly more mites than the lower fungus concentration or the water-treated control. At the untreated control the most mites were alive (Table 2).

Surprisingly, after 7 days the number of broad mites in the treatment with the higher rate of fungus suspension had increased tremendously, and was now not different from the number of live mites treated with the lower fungus rate. Still, the numbers of mites alive after 7 days were significantly lower in the fungus treatments than in the two control treatments.

Discussion

Metarhizium anisopliae CKM-048 was the most virulent fungal strain, hence the most promising candidate for controlling broad mite larvae and adults. Also *B. bassiana* CKB-048 showed good efficacy against larvae (95% mortality) and it was the second best against adults (ca. 50% mortality), so this may be considered another effective microorganism against broad mites, worthy of further investigations.

The concentrations of *M. anisopliae* CKM-048 needed for killing 50% of broad mite larvae and adults (8.71×10^6 and 1.32×10^7 conidia/ml, respectively), were not far apart. Yet, the time needed for killing 50% (when treated with 2×10^8 conidia/ml) of adults (3.4 days) was clearly longer than the time needed for killing half of the larvae (2.4 days). This might point at a higher tolerance of adults compared to larvae. Mite eggs were not found to be infected by any of the fungal isolates—perhaps the duration of the egg stage (1–2 days) is simply too short for the fungal mycelium to cause harm.

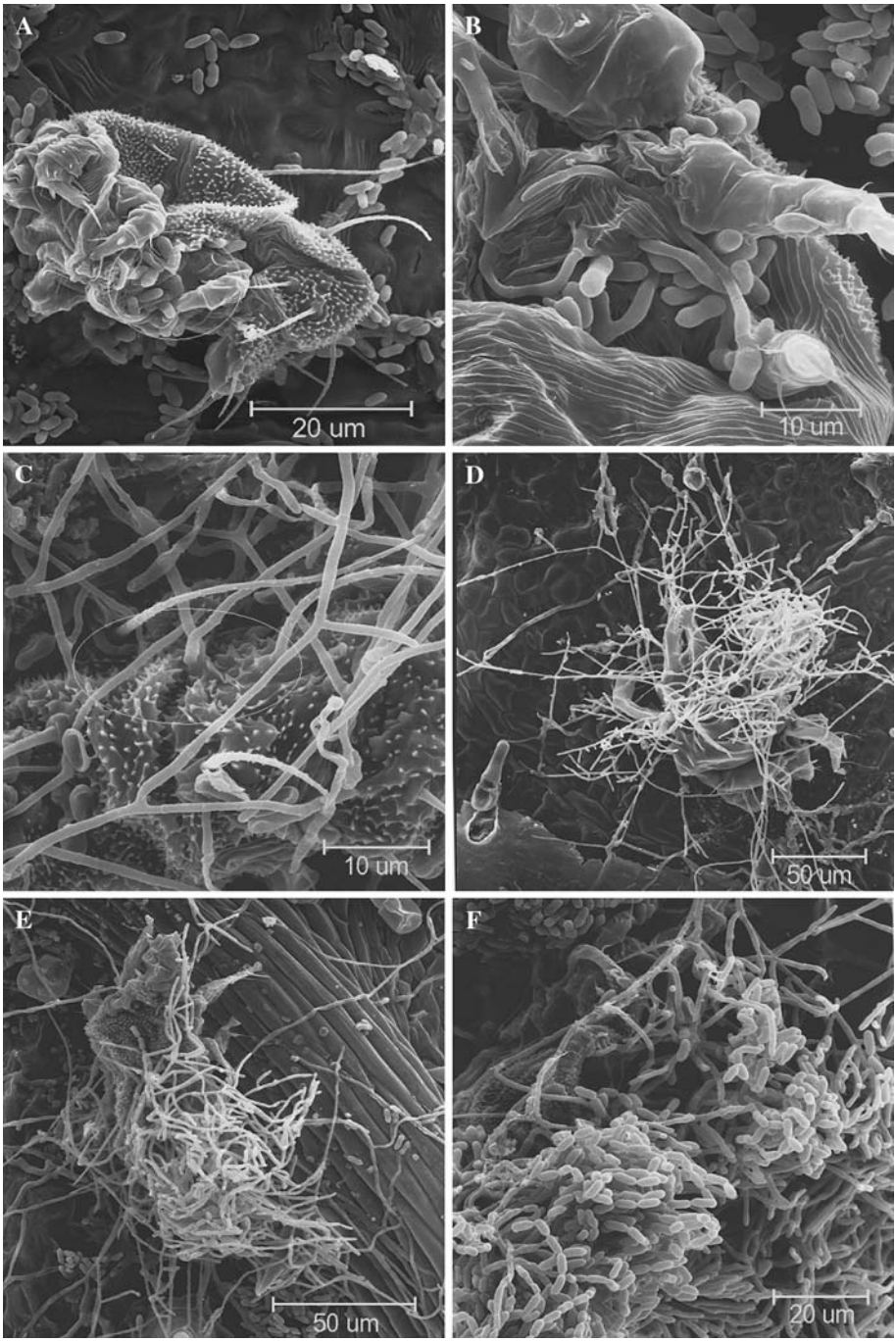

Fig. 5 A series of SEM pictures showing infection steps of *Metarhizium anisopliae* CKM-048 on the broad mite. (**A**) Conidia adhere on the broad mite's cuticle at 1-2 h after contact. (**B**) Germination from conidia at about 48 h after contact. (**C**) Penetration into the broad mite's body about 60 h after contact. (**D**) Extruding of fungus from the broad mite's body about 72 h after contact. (**E**) Colonization over the broad mite's body about 96 h after contact. (**F**) After 120 h, fungus started its conidiogenesis stage

Table 2 Mean number of broad mites (±SE) surviving per mulberry leaf up to 7 days after treatment

Treatment	Number of moving broad mites per leaf*			
	Before spray	1 day	4 days	7 days
Amitraz 20% w/v	26.40 ± 0.75^{ns}	0.05 ± 0.03^{b}	0^{d}	0^{d}
M. anisopliae CKM-048 2×10^8 conidia/ml	25.73 ± 0.92	23.67 ± 0.36^{a}	19.13 ± 2.76^{c}	30.97 ± 1.95^{c}
M. anisopliae CKM-048 2×10^7 conidia/ml	25.83 ± 0.78	23.65 ± 0.81^{a}	32.43 ± 1.51^{b}	33.80 ± 0.49^{c}
Control (treated)	26.73 ± 0.31	24.00 ± 0.43^{a}	37.18 ± 1.28^{b}	38.80 ± 1.08^{b}
Control (untreated)	25.80 ± 0.74	23.99 ± 1.03^{a}	47.82 ± 1.52^{a}	52.28 ± 2.38^{a}
% Coefficient of variation	7.29	9.48	17.21	13.39

* Means followed by the same letter within a column did not differ significantly ($P > 0.05$, DMRT)

The finding of enzyme activity confirmed the fungus' modes of action, because both protein- and chitin-digesting enzymes would be needed for lysis of the mite's cuticle, prior to mycelia penetration. Furthermore, the microscopic evidence underpinned the fungal infection of the mite.

The greenhouse assay suggested that *M. anisopliae* CKM-048 has a fairly short persistence on mulberry leave. Therefore, re-application of *M. anisopliae* CKM-048 within 3–4 days will be necessary. The application of microbial pesticides needs to be repeated especially for short-life-cycle pests, such as broad mites. Broad mites not only have a short life cycle, they are also parthenogenetical (Gerson 1992), and they are known to use insect hosts, specifically some whiteflies species, to move phoretically from plant to plant (Palevsky et al. 2001)—both factors will favour their potential rapid re-emergence.

Broad mite has not yet developed resistance to amitraz. However, *M. anisopliae* CKM-048 could be considered as an alternative tool for broad mite control in an integrated pest management program.

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