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INFECTION PROCESS OF ENTOMOPATHOGENIC FUNGI *Metarhizium anisopliae* IN THE *Tetrancyhus kanzawai* (KISHIDA) (TETRANYCHIDAE: ACARINA)

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ABSTRACT

The importance of fungal attack to acarina has been observed but not yet on *Tetraychus kanzawai*. When infected mite, either within in a same species or even in a different species of entomopathigenic probably have similar signs and simptoms to mites. Characteristic of entomopathogenic fungus to *Tetranychus kanzawai* was investigated. Three selected isolates of *Metarhizium aniopliae*, from Philippines and Indonesia were evaluated. The following aspects were investigated: (1) Bioassay tes of virulence, (2) Koch Postulate and (3) Infection process on each fungus against mite. Virulence of Metarhizium anisopliae was 1.4 X 10^3, 7.2 X 10^2 , 5 X 10^2 PIB per ml on Ma4, Ma5 and Ma6, respectively. On Koch Postulates, Reinoculation and reisolation of all seven isolates from infected *T. kanzawai* showed definite signs of infection. This demonstrated its entomopathogenic capacity. In this experiment, adult mites were sprayed with 10^8 per ml concentrations of conidia observed under light microscope and Scanning Electron Microscopy (SEM). The result found *T. kanzawai* was very susceptible to three isolates *M. anisopliae.* In the end of the trials, fungal growth was detectable as early as 2 until 4 days of observation. Infection process with microphotograph and Scanning Electro Microscopy showed attachment, germination and penetration, extrusion and conidiogenesis fungal form.

Keywords: Infection, *Tetranychus kanzawai*, *Metarhizium anisopliae*, Light microscopy and Scanning electro Microspopy (SEM)

INTRODUCTION

Entomopathogenic fungi were among the first organisms to be used for the biological control of pests. More than 700 species of fungi from around 90 genera are pathogenic to insects. Most are found within the Class Deuteromycetes and Order Entomophorales (Zygomycetes). Some entomopathogenic fungi such as *Aschersonia aleyrodis* (Webber) have restricted host ranges and infect only scale insects and whiteflies. Other fungal species within Hypomycetes have a wider host range, with individual isolates being more specific, such as *Metarhizium anisopliae* (Metschnikoff) Sorokinand *Beauveria bassiana* (Balsamo) Vuillemin**.** Numerous studies have been conducted on these fungal isolates which have been well characterized with respect to pathogenicity to several species of insects. These generalist fungi have also been observed on mites and can be developed as potential biocontrol agents against major mite pests of agricultural crops (Chandler *et al.*, 2000) such as the spider mite, *Tetranychos urticae*.

Fungal pathogens such as *Beauveria, Metarhizium, Paecilomyces* and *Verticillium* have also been used to control spider mites. Alves *et al*.. (2004) mentioned the potential to control *Tetranychus urticae* (Koch) (Tetranychidae: Acina), a major mite pest, using the entomo-pathogenic fungus of *B bassiana* (Bals) yeast phase spraying suspensions that composed of 10^5, 10^6,10^7 and 10^8 conidia/ml caused mortality up to 77.8 %. This research has also been elucidated by Barreto *et al.* (2004) who experimented with *B. bassiana* and *M.anisopliae* which were bioassayed for their lethal effects on

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the green mite, *Mononychellus tanajoa* (Bondar) on 3.5 cm cassava leaf discs. Suspensions standardized at 10^8 conidia per mL· diameter were inoculated with the pathogen by immersion in 20 mL of the suspension for 5 seconds. The result showed that Lethal Concentrations (LC50) of 3.93×10^6 conidia per mL and 7.44×10^8 conidia per mL were determined for *B. bassiana* and *M. anisopliae*, respectively. *B. bassiana* isolate 645 was the most efficient, being an alternative for use in biological control programs against the cassava green mite.

One of succesful of biological control program is time rate to kill the insect. The test due to fungal attack process is important. The importance of fungal attack development was related to the effectiveness of this entomo-pathogenic fungi. This study aims to observe the infection process of *M. anisopliae* selected from Indonesia and Philippines (collected from the Insect Pathology Laboratory of the Crop Protection Cluster, UPLB) to *Tetranychus kanzawai*.

MATERIALS AND METHODS

Preparation of Concentrations

Conidia were counted by using improved Neubauer Haemocytometer under binocular microscope at 400x magnification. The counts were replicated three times. The total amount of conidia was counted using the formula of Hansen (2000) as follows:

Conidia/ml = total cell count in 5 C squares x 50,000 x dilution factor

Ten ml suspension of Phosphate Buffered Saline (PBS) 0.85% was poured into PDA bottles and shaken. The PBS composition was: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4 with pH of 7.4 containing 0.01 % Triton X-100. Conidium was released from PDA when the suspension turned the colour into turbid.

Scanning Electron Microscope (SEM)

Specimens of four-day old infected female adult mites were examined by SEM. Infected mites were immersed overnight in 2.5 % glutaraldehyde in PBS, pH 7.0 and washed 3 times with PBS at 10 minute interval for each wash. Washed samples were immersed in 1% osmium tetroxide (diluted in PBS) for 30 to 45 minutes. The osmium tetraoxide treatment was followed by 3 washes in sterile water at 10-minute interval per wash and dehydrated at room temperature in a graded series of 25 %, 50%, 75% and 95% ethanol with 30-minute interval for each step. The final step was followed by 3 changes of absolute grade ethanol, with two changes at 30 minute interval and overnight for the last 100% ethanol change. After dehydration, the samples were transferred into mesh microcontainers flooded with 100% ethanol for critical point drying. Critical point drying was done for 45 minutes. The dried samples were mounted onto pin stubs with double-sided tape in different orientations and spitter coated with gold coating. Samples were examined and images were taken using Hitachi variable pressure SEM in high vacuum mode. The process of adhesion, germination and colonization was observed by using light microscope and micro camera under 1000x magnification from a small amount of colony taken from the flask.

Bioassay Tests for Virulence

Laboratory Screening

Preliminary phase.

Ten adult female mites aged 24 to 36 hours after emergence from the stock colony were transferred onto abaxial surface of a 1.5 cm leaf disc in 2.5 cm petri dish with a brush. The females were classified visually by observation of the shape of the opisthosoma, which is round in females and funnel-shaped opisthosoma in males.

A series of 5 dilutions was prepared for each of the 14 fungal suspensions at 10^4, 10^5,10^6, 10^7, and 10^8 conidia per ml concentrations. PBS served as control. These suspensions were transferred into a plastic sprayer. Spraying was done first on the control mites using 0.5 ml of suspension and then followed by the fungal suspensions starting with the lowest up to the highest concentration. Six trials were conducted using 10 mites per concentration with 3 replications. Mortality percentage was observed at 5 days post infection (DPI).

Final phase.

The top 3 isolates of *M. anisopliae* from the preliminary phase including the lone *P. lilacinus* were further subjected to LC_{50}

determination using the same procedure but with 8 concentrations from 10^1 to 10^7 conidia per ml. Mortality data obtained at 5 DPI were subjected to LC_{50} analysis. Six trials were conducted using 10 mites per concentration with 3 replications.

Koch's Postulates

Pathogenicity tests were carried out for the three entomopathogenic isolates in 2 stages, namely: reinoculation and reisolation for each isolate. Adult female mites were sprayed with 10^8 conidia ml^{-1} of a fungal isolate. Three replicates were carried out for each isolate. Control mites were sprayed with 0.85% PBS. Observations were conducted daily until a sufficient number of dead adults have been reinoculated. The fungus which grew on female adults was reisolated in PDA and was inoculated back to other healthy adult females for infection. Further examinations were done on the characteristics of the spores and conidiophore isolates of infected female mites by fungi and the results were compared with the initial isolates that have been obtained under binocular microscope.

RESULTS AND DISCUSSION

Infection Process

Entomopathogenic fungi invaded their hosts by penetrated of the host cuticle. There are different characterirtics for the three isolates of *M. anisoplia.* After application of *M. anisopliae*, the conidia stuck to the integument on conidial germination processes (Figures 1a, 2a, and 3a).

Following the attachment, the next important stages in pathogenesis were germination and germ tube formation, as conidia on the surface of a host may suffer from desiccation, antibiosis from saprophytic microbes or removal with the old cuticle when moulting occured. As shown in Figure 2 b, 3b and 4b conidia germinated in response to a range of exogenous carbon and nitrogen sources (St. Leger *et al.,* 1993). There is variation in germination between different strains, and which may be correlated to host species. *M. anisopliae* var*. anisopliae* and var*. majus* isolated from scarabeids germinate at high frequency only in the presence of a crude protein/chitin product. Those isolated from other Coleopteran species germinate and form appressoria in yeast extract at 0.0125% (St. Leger *et al.,* 1998). The appressorium represents an adaptation for concentrating physical and chemical energy over a very small area so that ingress may be achieved efficiently. Thus, formation of the appressorium plays a pivotal role in establishing a pathogenic interaction with the host. Appressorium formation may be influenced by host cuticle that indicate the involvement of the intracellular second messengers Ca^{2+} and cyclic AMP (cAMP) in appressorium formation (St. Leger *et al.,* 1993).

Conidia growth was found on the mite gnathosoma and anal regions, but some conidia were also saw on the leg. Germination tubes on the mite's cuticle were observed during the penetration process (Figure 2b, 3b and 4b). It was assumed that it might be due to the production of enzymes in the infection process.

Enzymes produced by the fungal mycelium digested the insect cuticle, triggering the penetration of the insect integument. In some areas such as the mite's head, extensive growth of germ tubes was observed, probably due to resistance to fungal penetration by having more sclerotized cuticle.

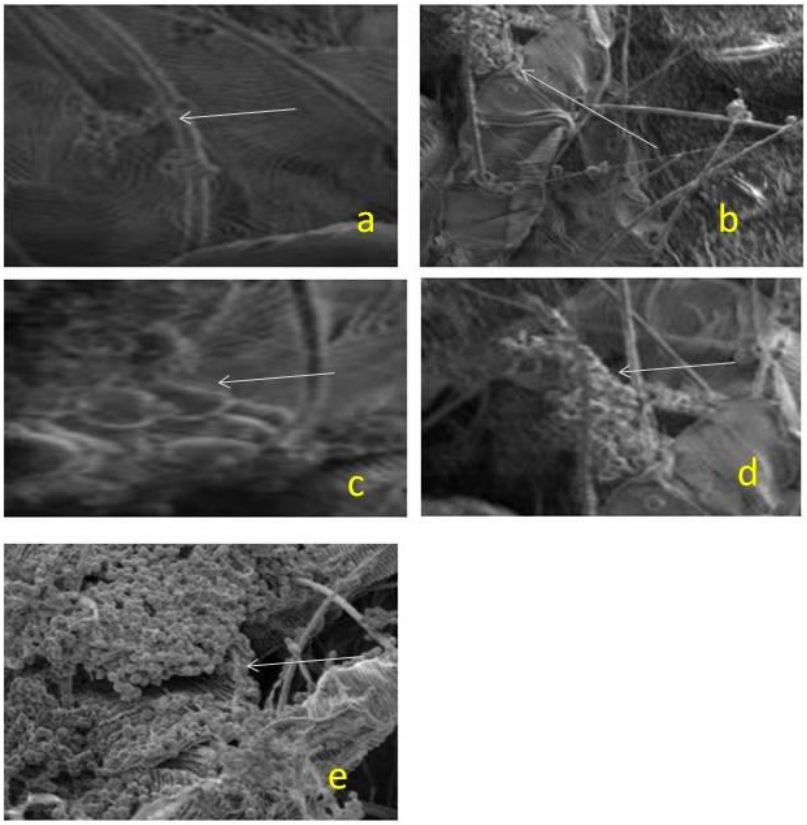

Figure 1. Microphotograph of *Metarhizium anisopliae* 4 infecting *Tetranychus kazawai* 4 days after inoculation by Scanning electron microscope micrographs (800 X). a) Conidia adhered to the tegument. b) Germinating conidium, with halo formation in the cuticle c) Penetration of a germ-tube into the cuticle d) Extrusion of the mycelium at intersegmental region e) Conidiophore formation and conidiogenesis on the mite thorax

Host colonization was observed to occur after 48 hours (Figure 1d, 2d and 3d). The mites killed by *M. anisopliae* had a white colouration which is a characteristic of oosporein activity common in insects infected by this fungus. It seems Bb6 was the most potential since it had colonization in 24 hours. Physical penetration has been shown during host invasion by some entomopathogen (Hu, 2005).

Enzymatic degradation of host cuticle may result from either extracellular or cell wall bound enzymes. Extracellular enzymes corresponding to the main chemical constituents of insect cuticle, protein, chitin and lipids, had been detected prior to 1986. However, there were only little information about types, modes of action, regulation, cellular localization, sequence and levels of production (St. Leger *et al.,* 1993)

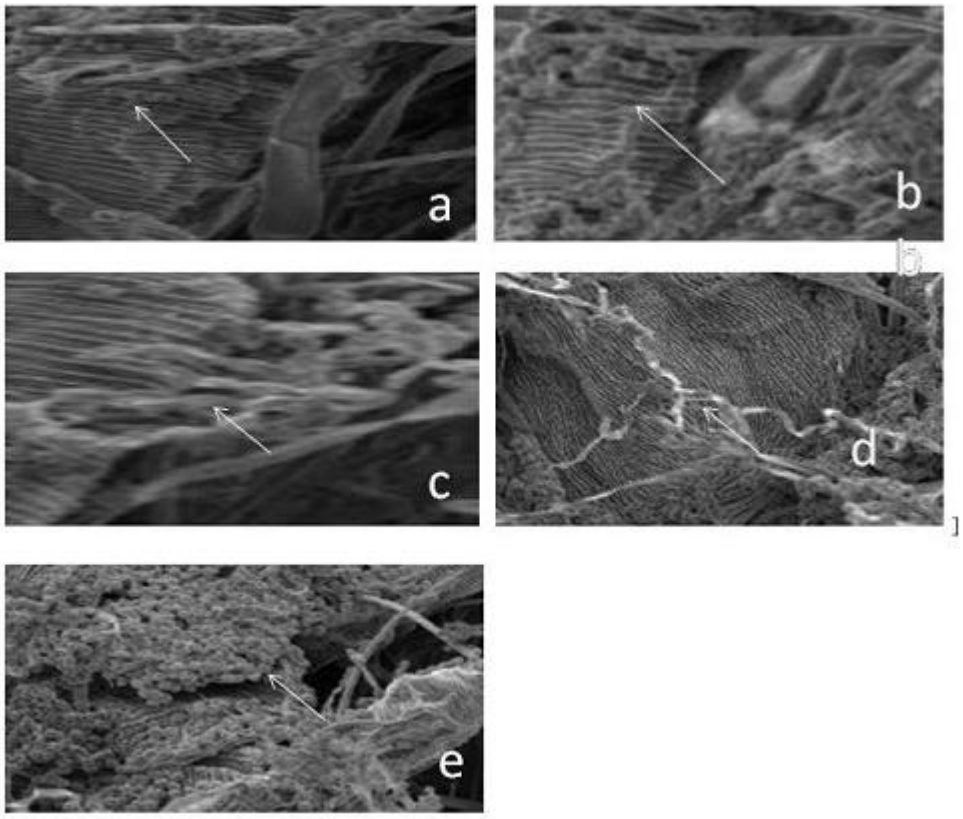

Figure 2. Microphotograph of *Metarhizium anisopliae* 5 infecting *Tetranychus kazawai* 4 days after inoculation by Scanning electron microscope micrographs (800 X). A)Conidia adhered to the tegument. b) Germinating conidium, with halo formation in the cuticle c) Penetration of a germ-tube into the cuticle d) Extrusion of the mycelium at intersegmental region e) Conidiophore formation and conidiogenesis on the mite thorax

Mycelial extrusion on cadavers occurred between 96 and 120 hours after inoculation, mainly in the intersegmental areas and other area for complete cuticle degradation. The process of conidiogenesis occurred between 96 and 120 hours after inoculation.

Pathogenic isolates of *M. anisopliae, B. bassiana*, and *V. lecanii* grown in buffered and unbuffered liquid cultures containing 1% ground cuticle as the sole carbon source produced a range of extracellular cuticle-degrading enzymes (St. Leger *et al.,* 1996 a). Major enzyme activities include endoprotease, chitinase and N-acetylglucosaminidase (NAGase). Treatment of cuticle with these enzymes showed that pre-treatment with protease enhanced chitinase activity at 3.5 fold, implying the shielding of cuticular chitin by protein (St. Leger *et al.,* 1998).

The sequence of enzymes produced in cuticle cultures by each species was similar. Esterase activity and enzymes of the proteolytic complex (protease, aminopeptidase and carboxy-peptidase) appeared first (< 24 hr) and increased rapidly after 28 hrs. Chitinase was produced at low basal levels for 3 to 5 days, after which it increased rapidly. Lipase was not detected until day 5 for any fungus tested (St Leger *et al.,* 1993). The early production of high levels of protease was considered an indication of their potentially important role in cuticle penetration.

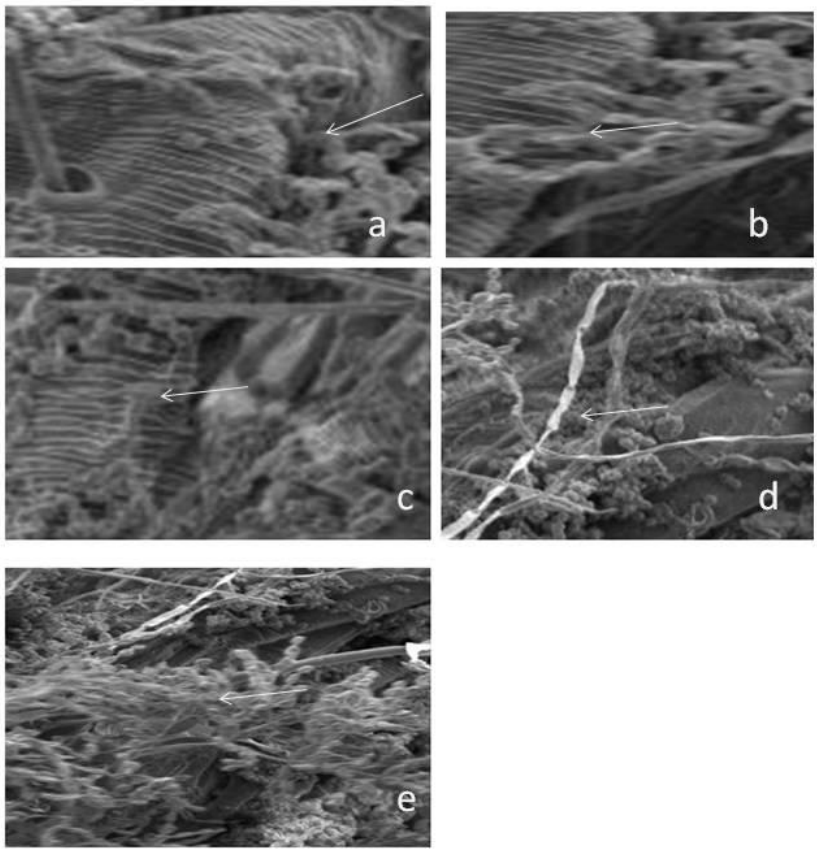

Figure 3. Microphotograph of *Metarhizium anisopliae* 6 infecting *Tetranychus kazawai* 4 days after inoculation by Scanning electron microscope micrographs (800 X). a) Conidia adhered to the tegument b) Germinating conidium, with halo formation in the cuticle c) Penetration of a germ-tube into the cuticle ; d) Extrusion of the mycelium at intersegmental region ; e) Conidiophore formation and conidiogenesis on the mite thorac.

Virulence Test

All isolates of *M. anisopliae* infected the mite *T. kanzawai* . They demonstrated rapid external hyphal development and sporulation under moist condition. Initially, hyphal strands emerged from the anal region of the mite cadaver and then quickly covered the cadaver with profuse hyphal growth followed by sporulation within 4 to 6 days.

M. anisopliae appeared greenish in colour. Among the seven *M. anisopliae* isolates, the most pathogenic at 5 DPI, the three most pathogenic isolates of *Metarhizium anisopliae* were Ma4, Ma5 and Ma6 from among all isolates (Table 1).

With regards to the performance variability of the different isolates, Sosa Gómez and Alves (1983) reported a high enzymatic activity in more virulent isolates of *M. anisopliae* from several Brazilian regions, and suggested that they are probably associated with the presence of enzymes that influence the penetration process of the fungus (St Leger *et al.,* 1988; De La Rosa *et al.,* 1997), as well as with toxins such as destruxins and beauvericin, present in *M. anisopliae* and *B. bassiana* respectively, which vary in different isolates (Roberts and St. Leger 2004). However, unlike insecticides, fungal infection takes 4-6 days after application to kill a mite. During this time the infected mite can cause serious damage to the crops (St. Leger *et al.,* 1996 b).

Table 1. LC_{50} values (conidia/ml) of 3 entomopathogenic fungi at 5 days after application on *T. kanzawai* adult females

species	isolate	lc_{50}	95% fiducial limits
M. anisopliae	Ma4	1.4×10^3	$6.6 \times 10^2 - 4.0 \times 10^3$
	Ma5	7.2×10^2	$3.1 \times 10^2 - 1.5 \times 10^2$
	Ma6	5.0×10^2	$2.3 \times 10^2 - 1.0 \times 10^3$

In the study of Tamai *et al.* (1998) on the mite *T. urticae* using *Beauveria* spp. isolates at a concentration of 5×10^8 conidia ml^{-1}, mortality ranged from 5.5 to 100% in total. Similarly, Oliveira *et al*. (2002), working with *B. bassiana* isolates at 10^8 conidia mL^{-1} and the red mite *Oligonychus yothersi* (McGregor), recorded 77 to 98% mortality.

More definite LC_{50} (Table 1) for selected fungus isolates were determined for *T*. *kanzawai* at 5 DPI. The order of decreasing pathogenicity for the isolates remained to be Ma6>Ma5>Ma4 .For this particular determination of LC_{50}, a total of 8 concentrations was used and the mortality values ranged from 10 to 100%, showing a quite high reliable of this research.

The result is similar with Maketon *et al*. (2008) using *M. anisopliae* CKM-048, *M. anisopliae* was the most virulent fungal strain, hence the most promising candidate for controlling broad mite larvae and adult of *Polyphagotarsonemus latus.* Also, *B. bassiana* CKB- 048 showed good efficacy against larvae with 95% mortality and it was also effective against adults (ca. 50% mortality), so this may be considered another effective microorganism against broad mites. 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 per ml, respectively) were not far apart. Yet, the time needed for killing 50% (when treated with 2×10^8 conidia per ml) of adults (3.4 days) was clearly longer than the time needed for killing half of the larvae (2.4 days).

Koch's Postulates

Reinoculation and reisolation of all three isolates from infected *T. kanzawai* showed definite signs of infection. This demonstrates its entomopathogenic capacity (Figure 4).

T. kanzawai, when infected with *Metarhizium anisopliae* Ma4, Ma5 and Ma6, showed mycosis on the body surface of the mites with the appearance of green mycelia. Mycelia on the mites' body were formed between 4-6 days. Conidiophores of three *Metarhizium* isolates aggregated in dense tuft with repeated, more or less, verticillate branching on Ma4 and Ma6, with Ma5 having the most tufts.

CONCLUSIONS

The three most pathogenic isolates of *M. anisopliae* (Ma4, Ma5, a6) showed attachment, germination and penetration, extrusion and conidiogenesis fungal form. M6 was the most potential only need colonization on 24 hours. The infection process was supported by toxicity value of LC_{50} for selected fungus isolates: Ma6>Ma5>Ma4 repectively. Also by Koch's test on *T. kanzawai*, when infected with *Metarhizium anisopliae* Ma4, Ma5 and Ma6, showed green mycelia on the body surface of the mites as well as showed on infection process experiment.

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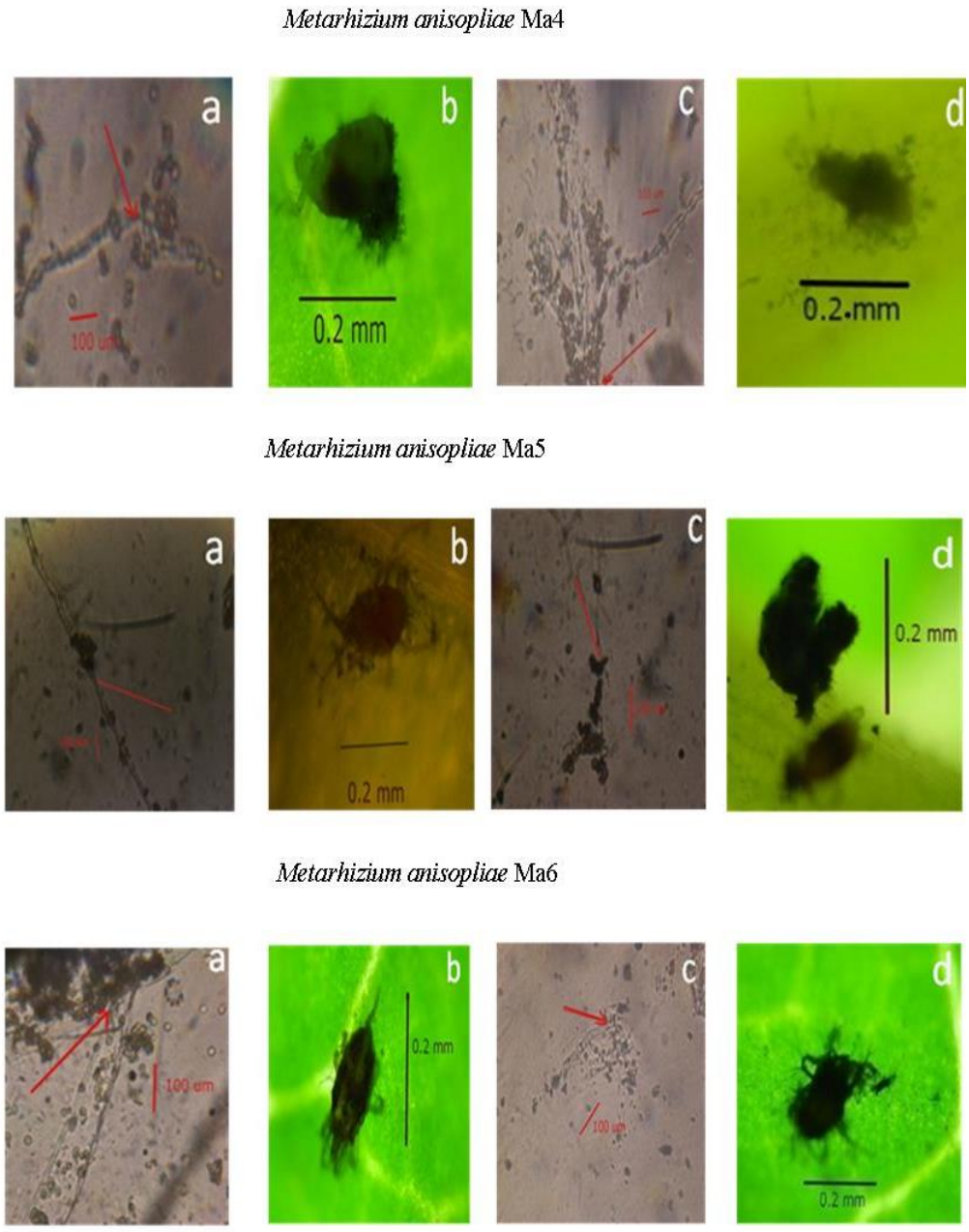

Figure 4. Disease-pathogen associations of Koch's Postulate on *Tetranychus kanzawai* by entomopathogenic fungi *M. anisoplae* (400 x). Reinoculation of fungus (a), infected from reinoculation (b), reisolation of fungus (c), infection of mite's from reisolation (d)

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