INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 7, No. 3, p. 16-23, 2015

RESEARCH PAPER OPEN ACCESS

Role of new novel entomopathogenic fungi *Paecilomyces lilacinus* to mortality and infection process of *Tetrancyhus kanzawai* (Kishida) (Tetranychidae: Acarina)

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Key words: *P.lilacinus*, Mortality, Characteristics, SEM, Infection Process.

http://dx.doi.org/10.12692/ijb/7.3.16-23 Article published on September 22, 2015

Abstract

P. lilacinus is a common soil hyphomycete with a cosmopolitan distribution. Since Its characteristic has similarity with Metarhizium and Beauveria, it is assumed that it can be effective as well Metarhizium and Beauveria . The experiment was conducted in the Green house with papaya plant. The papaya seedlings were hand-sprayed with suspension with a density of 1 x 10^6, 1 x 10^7, 1 x 10^8 conidia ml^{-1}, with 3 replications. Adult mortality of Tetranycus kanzawai was observed daily with 10 x hand magnifying glass until 10 days of 100% mortality was reached (based on preliminary research), after treatment. Second experiment Scanning Electron Microscope (SEM) to know charactreistic of *P.lilacinus* and indection cause by its fungi.The third experimet was morphological characterization of fungi. The observations covered color and mycelia structure. Furthermore shape, conidia, and conidiophores were observed under binocular microscope and microcamera under 1000 x magnification from a small amount of colony taken from the flask. The result showed that on the concentration of 10^7 it cause mortality of *T. kanzawai* 70 %, while on 10^8 was 74,44%. The P.lilacinus characteristic that it has pink color, The color of young *P. lilacinus* colony was white, but when sporulating it changed to various shades of vinaceous. *P. lilacinus* formed a dense mycelium, bore phialides from the ends of which spores were formed in long chains. The reverse side is suncolored. Conidia were in divergent chains, ellipsoid to fusiform in shape, and smooth-walled to slightly roughened.The infection proses showed Conidial adhesion to the integument happened immediately 12 to 24 hours after inoculation and formed conidial germination Penetrations forming appressoria were also obserbed in *P. lilacinus* , characterized by a thickening of the extremity of the germ-tubes as well as extrusion and conidiogenesis which were almost similar with *M. anisopliae* and *P. lilacinus*.Conidial adhesion to the integument happened immediately 12 to 24 hours after inoculation , and formed conidial germination (Figure 3b). Conidial adhesion to the integument happened immediately 12 to 24 hours after inoculation (Figure 3a), and formed conidial germination .

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Introduction

Tetranychus kanzawai (kishida) is an important mite pest throughout East and Southeast Asia, attacking over a hundred species of plants, including many crops and ornamental plants (Takafuji et. al, 2005). It is normally an outdoor species, but can attack greenhouse plants as well. The eggs are often laid on the undersides of leaves. They are spherical in shape and are clear when freshly laid. The larvae and nymphs are yellowish green, and the adults are red or yellowish red depending on host plants. They often feed on chloroplasts on the under surface of the leaf, which causes the upper leaf surface to develop characteristic whitish or yellowish stippling. As mite feeding continues, the stippling coalesces to form brownish lesions (Cheng, 2002). Heavy damage eventually leads to wilting and defoliation, which further reduces plant growth.

Spider mite (Tetranychidae) is widely distributed worldwide, including in the Philippines and Indonesia. The most common Tetranychid mite as pest in the Philippines and Indonesia are *Tetranychus kanzawai* and *T. urticae*. *T. kanzawai* is important throughout East and South Asia and is a polyphagous mite. In the Philippines, it commonly infests cassava and papaya plants (Guavarra, 1981) as well as hundreds of plants that include vegetables and food crops such as strawberries, peppers, tomatoes, potatoes, beans, and corn. The mites attack and severely damage the older leaves of papaya and sometimes, its seedlings. Its serious damage causes the leaves to dry up, thus, reducing the photosynthetic activity of the plant.

Some tactic that needed to control mites such as culural practices, biological using predators and Entomopathogens. Entomopathogenic fungi is an important component in Integrated Pest Management (IPM). There are some advantages in using them. Firstly, they are important natural enemies of arthropods (Chandler *et al.*, 2000) capable of infecting them directly through the integument; secondly, cultivation of those fungi and production of infective conidia are easy and fairly cheap (Roberts and Krasnoff, 1998); and finally, entomogenous fungi can be found under different ecological conditions (Ferron, 1984).

Another candidate of member of Deuteromycetes is *Paecilomyces lilacinus* which is effective for control of cyst and root-knot nematodes in greenhouseand the open field condition. There are still lack information about the effectiveness of this fungi to insect pest. Some reseachers that work with the fungus *P. lilacinus* , Borisov and Ushchekov, 1997 found that *P. lilacinus can* reduce population of the tomato leaf-miner, While Fiedler and Sosnowska (2006) first report on efficacy of this fungus in whitefly control in Poland. Sosnowska (2003) stated that *P. lilacinus* was effective against this pest even at low temperature (10^{o}C). This feature makes it possible to apply the fungus at low temperatures. *P. lilacinus* is a common soil hyphomycete with a cosmopolitan distribution (Samson, 1974). Since Its characteristic has similarity with metarhizium and Beuveria, it can be assumed that it can be effective as well. The main objective of the research presented in this paper was to evaluate the pathogenicity *P. lilacinus* in control of *Tetranychus kanzawai*.

Materials and methods

Time and Place of Study

The study was conducted at the Insect Pathology Laboratory and Entomology Green House of the Crop Protection Cluster, University of the Philippines Los Baños from February 2010 to March 2011.

Green House Bioassay

The isolates were tested on mites under green house condition using treated papaya seedlings in a completely randomized design. 60-days old papaya seedlings (Solo variety) were prepared and each was infested with 10 adult mites using fine brush. After sticking the mites using tangle foot, the papaya seedlings were covered with a plastic box. Seven selected isolates were prepared in a suspension consisting of 20 ml distilled water. The papaya seedlings were hand-sprayed withs suspension with a density of 1 x 10^{6}, 1 x 10^{7}, 1 x 10^{8} conidia ml^{-1}, with 3

replications. Adult mortality was observed daily with 10 x hand magnifying glass until 10 days of 100% mortality was reached (based on preliminary research), after treatment.

The percentages of adult female mortality of *T. kanzawai* were calculated using the following formula:

$$\text{percent of mortality} = \frac{\text{total mites mortality}}{\text{total mites test}} \text{ x } 100$$

If the control has some mortality below 10 %, the data will be corrected using the Abbott Formula:

$$Pt = \frac{Po - Pc}{100 - Pc} \text{ x } 100$$

Where:
Pt = % mortality after correction
Po = % mortality of adult mites after treatment
Pc = % mortality of adult mites on control.

The mite mortality data were submitted for analysis of variance using F test and the means were compared by Duncan multiple range test (P<0.05) using the SAS software package.

Scanning Electron Microscope (SEM) of Infection proses of P. lilacinus to T.kanzawai

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 intervals for each wash. Washed samples were immersed in 1% osmium tetroxide (diluted in PBS) for 30 to 45 min. The osmium tetraoxide treatment was followed by 3 washes of 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.

Morphological characterization of fungi

The observations covered color, mycelia structure, colony shape, and growth rate. Furthermore shape, conidia, and conidiophores were observed under binocular microscope and microcamera under 1000 x magnification from a small amount of colony taken from the flask.

Result and discusssion

Efficacy of Selected Entomopathogenic Fungi in the Greenhouse

The mortality of *T. kanzawai* sprayed with the selected entompathogenic fungi are presented in Table 1.

Table 1. Percentage mortality of *Tetranychus kanzawai* exposed to papaya seedlings sprayed with different conidial suspension of entomopathogenic fungi at 10 days after treatment under green house conditions.

Fungal isolates	Mean percentage mortality		
	Conidia ml^{-1}		
	1.0 x 10^6	1.0 x 10^7	1.0 x 10^8
Bb4	58.89^{cd}	72.22^{d}	82.22^{d}
Bb5	65.56^{abcd}	80.00^{bc}	87.78^{c}
Bb6	73.33^{ab}	84.44^{bc}	95.56^{ab}
Ma4	6.33^{bcd}	78.89^{c}	90.00^{c}
Ma5	71.11^{abc}	85.56^{ab}	92.22^{bc}
Ma6	76.67^{a}	91.11^{a}	98.89^{a}
Pl	53.33 ^{d}	70.00^{d}	74.44^{e}
Control	0^{e}	0^{e}	0^{f}

* Means with the same letter are not significantly at DMRT 0.05; n=960; df = 7.

Percentage mortality by spraying with 1.0 x 10^6 to 1.0 x 10^8 conidia ml^{-1} of seven isolates of entomopathogenic fungi after 10 day ranged from 53.3% to 98.9 %. The results obtained among the seven isolates were significantly different. The result was showed that *P.lilacinus* also caused *T. kanzawai* mortality eventhough Ma6, Bb6 and Ma5 were not significantly different and were the highest among 7 isolates. Data presented in Table 1 also indicate that using higher concentrations of conidia per ml enables the isolates to cause higher mortality to *T. kanzawai*.

The findings of the three trials show that the epizootic of entomopathogenic fungus can regulate the population of mites. Although it is not originally from mites, the fungal can be pathogenic to *T. kanzawai*. Steinhaus (1963) reported that fungal isolates from non-acarine hosts were pathogenic to *Varroa destructor* Anderson and Trueman.

Peña *et al.* (1996) found that fungal isolates originating from *Polyphagotarsonemus latus* Banks (Tarsonomidae) were more pathogenic than those isolated from other hosts. Strict adaptation of *M. anisopliae* strains to the original host, though, has been likewise reported in the case of scarabaeid beetles (Ferron *et al.,* 1972).

Study 2. Characterization of the Entomopathogenic Fungi

The description and characteristics of the colony and mycelia of the seven fungal isolates on PDA medium are presented in Table 2.

Table 2. Cultural characteristic of the selected entomopathogenic fungi virulent to *Tetranychus kanzawai.*

Isolate	Color		Mycelia structure	Colony Shape mean	Growth rate Mm	Size (mm)	No. of days to sporulation	Shape of Conidia	Size of Conidia (µM)
	Top	Bottom							
Pl	Pinkish	White	Thick and adpressed	Round	0,19	20	4	ovoid	4 x1.5

The color of young *P. lilacinus* colony was white, but when sporulating it changed to various shades of vinaceous. (Table 2). *P. lilacinus* formed a dense mycelium, bore phialides from the ends of which spores were formed in long chains. The reverse side is suncolored. Conidia were in divergent chains, ellipsoid to fusiform in shape, and smooth-walled to slightly roughened (Figure 1).

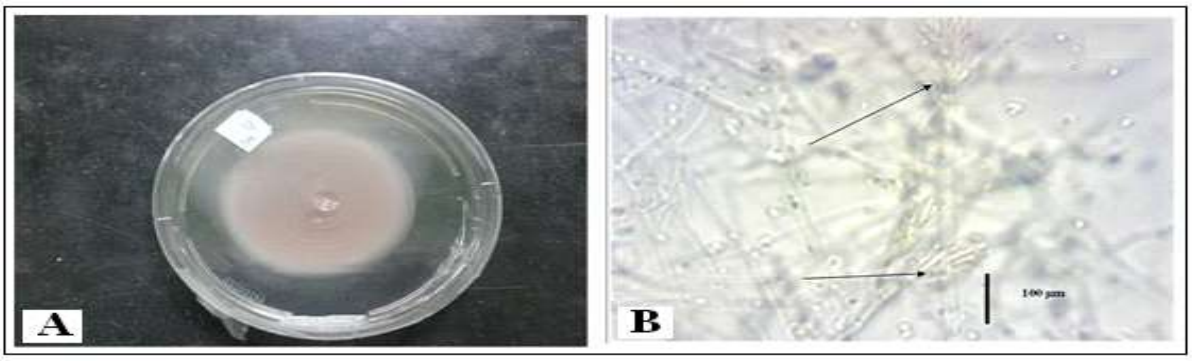

Fig. 1. Conidiophore and colony characteristic of *Paecilomyces lilacinus* (a and b). Arrows indicate the conidia and conidiophore.

The conidophore and phialides were swollen at their bases and tapered towards their apices. The conidia were globose and measured 2 to 3 µm at 1000 magnification (Figure 2).

Infection Process of Peacilomyces lilacinus

The infection process of *Paecilomyces* has many similarities with the events reported for *M.anisopliae, B. bassiana* and *P. lilacinus*. Conidial adhesion to the integument happened immediately 12 to 24 hours after inoculation (Figure 3a), and formed conidial germination (Figure 3b).

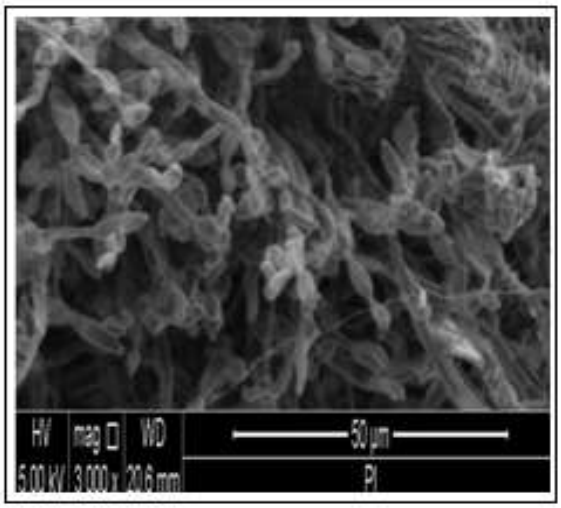

Fig. 2. Morphological isolates of *Paecilomyces lilacinus* showing the conidia and mycelium under scanning electron microscopy. The magnification and the scale bars are indicated at the lower part of the figures.

Penetrations forming appressoria were also obserbed in *P. lilacinus* (Figure 3 C), characterized by a thickening of the extremity of the germ-tubes as well as extrusion and conidiogenesis which were almost similar with *M. anisopliae* and *P. lilacinus* (Figure 4).

P. lilacinus infection takes place through integument and has a simple life cycle with no known sexual stage. It produces asexual spores called conidia. The spores germinate on the host cuticle and the hyphae penetrate into the hemocoel using enzymes and mechanical pressure.

Inside the hemocoel, the fungus assumes a yeast like phase, multiplying rapidly by budding or hyphal fussion (Tanada and Kaya, 1993). The death of the host is due to extensive mycelia colonization or toxin released during the yeast phase. The host's cadaver dessicates as the hypae deplete the nutrients and water for fungal development. The hyphae of the fungus eventually breakthrough the surface of the cuticle of dead insects

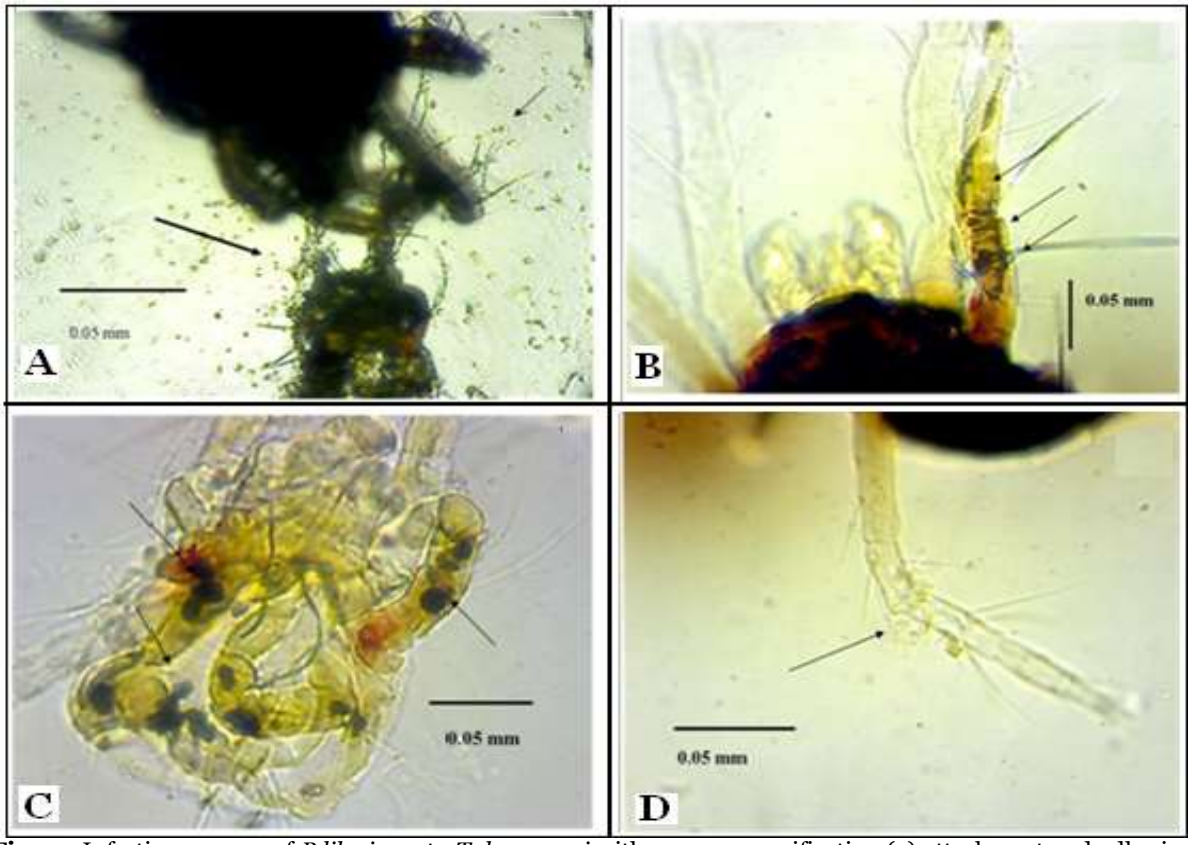

Fig. 3. Infection process of *P.lilacinus* to *T. kanzawai* with 1000 x magnification (a) attachment and adhesion after 24 h, (b) germination after 48 h, (c) colonization from 48 to 72 h on ophistosome, and (d) colonization from 48 to 72 h on intersegmental of leg. Arrows indicate the location of the fungus.

Mechanism of infection of *P. lilacinus* is initially through the adhesion of spores to the host cuticle. Adhesion involves the chemical and physical interaction of the insect's cuticle and spore surface. Spore attachment to host cuticle is a two stage process. In the first stage nonspecific adhesion brings the spore in this close contact with integument and allow the spore to remain long enough for the second stage to occur. The non specific attachment of Hypomycetes entomopathogenic fungal conidiophores to the insect cuticle is mediated by the hydrophobic interaction on the insect epicuticle and the conidial cell walls. Boucias *et. al* (1988) found that the outer surfaces of the conidia contain resilient layers of organized rodlets and it was shown that extracted *P. lilacinus* the same hydrophobic properties as the intact of conidia. Lectin-like association between spore surface antigens and host cuticle mediate the second stage of attactment.

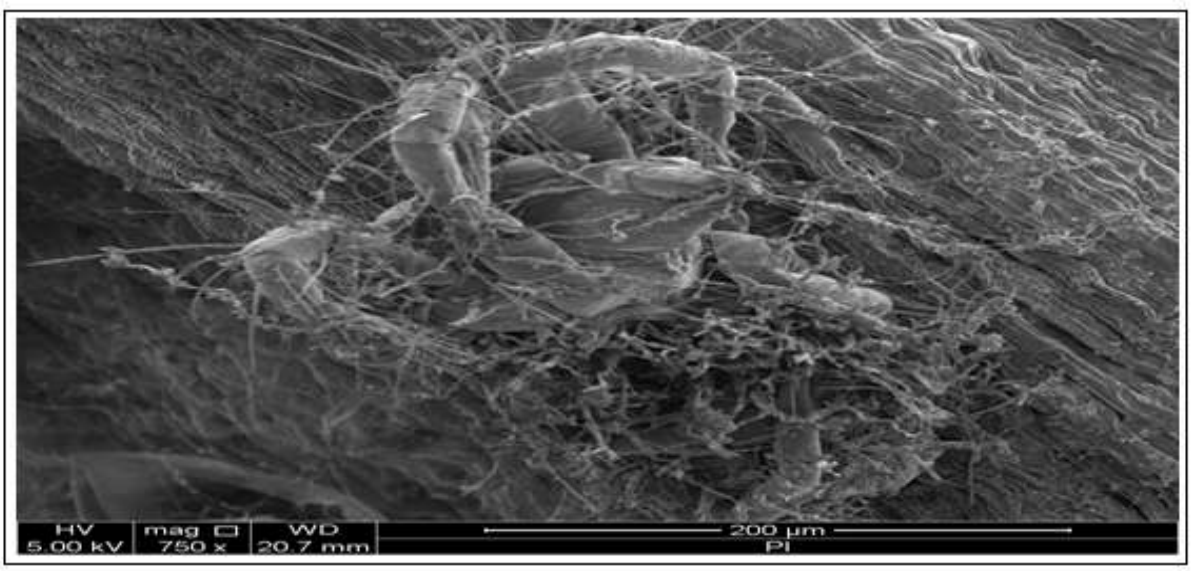

Fig. 4. Scanning electron micrograph of *Paecilomyces lilacinus* infecting *Tetranychus kanzawai* 4 days after infection (1000 x).

The next step in in spore germination. It is generally accepted that many fungal spores required a high relative humidity (RH), 90 % or greater for germ tube formation. found that it is needs a protein supply for germination, but sugar are not necessary. The germinating conidia, however, require utilizable of carbon, such as glucose, glucosamine, chitin, starch and nitrogen for hyphae growth (Tanada and Kaya, 1993).

Penetration of the cuticle involves both mechanical and chemical activities. Tanada and Kaya (1993) observed that when integument is hard, thick or smooth the germ tube of the fungus produces an appresorium that adheres to the cuticle with mucoid substances. The appresorium may form a penetration plate, penetration tube or hyphae and hyppal body for invasion. In this case, cuticle degrading proteases are secreted by appresorium on the cuticle surface and by the penetrant hyphae within cuticle. The production of chitinases, lipases, and proterases as been shown in vitro (St Leger, 1993). Changes in curvature of the cuticle had been documented suggesting that penetration pegs are applying mechanical pressure on the cuticle.

Growth of fungus in the hemocoel is the next step in infection of the host. The fungus retaliates against insect's defense mechanism by rapid production. This is usually accomplished by hyphal fission production separate hyphal bodies. Moreover, the fungus also produced toxins as counter measures against the host's body defenses. The later stages of mycosis induce physiological symptoms such as convulsion

and lack of coordination. Some infected exhibit abnormal behavior, such as lafty position, Dying towards the sun, climbing trees or foliage, affix themselves to an apex of plant. These behavioral alteration facilitate spore dispersal and are incredibly adaptive for the fungus.

Eventually, all organs of the insect are consumed and replace with hyphae. Under favorable conditions, hyphae repenetrate the cuticle and produce conidiophores outside the host. Resting spore is also produced both inside and outside of the cadaver (Boucias and Pendland, 1992).

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