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Acaricidal activities of whole cell suspension, cell-free supernatant, and crude cell extract of *Xenorhabdus stokiae* against mushroom mite (*Luciaphorus* sp.)*

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Abstract: *Xenorhabdus* bacterium has been used as a biological control agent against *Luciaphorus* sp., a mushroom mite endemic in Thailand. To develop an effective formulation of *Xenorhabdus stokiae*, treatments using different parts of *X. stokiae* isolate PB09 culture, including whole cell suspension, cell-free supernatant, and crude cell extract, were performed. The results show that different parts of *X. stokiae* isolate PB09 culture could induce variable effects on mite mortality and fecundity. Application with cell-free supernatant of *X. stokiae* culture resulted in both the highest mite mortality rate [(89.00±3.60)%] and the lowest mite fecundity [(41.33±23.69) eggs/gravid female]. Whole cell suspension of *X. stokiae* isolate PB09 culture was found to be slightly less effective than its cell-free supernatant, suggesting that *X. stokiae* was more likely to release its metabolites with acaricidal activities to the surrounding culture media. Crude cell extract of *X. stokiae* was not effective against mites. Cell-free supernatant of *X. stokiae* isolate PB09 was the most effective biological control agent and it could be conveniently used in future formulations instead of live bacteria.

Key words: *Xenorhabdus*, *Luciaphorus*, Whole cell suspension, Cell-free supernatant, Crude cell extract, Mushroom mite
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1 Introduction

Luciaphorus sp. (Acari: Pygmephoridae) is a mushroom mite that has become one of the major threats to the large-scale cultivations of several mushroom species, including *Lentinus squarrosulus* (Mont.) Singer, *Lentinus polychrous* Lev., Auricularia auricula-judae (Bull.) Wettst., and *Flammulina velutipes* Karst. (Bussaman *et al*., 2004). The use of insecticides, such as carbamates and organophosphates, to control this mushroom mite has had little success, and growers can only manage this pest by maintaining the recommended routine horticultural hygiene procedures (Bussaman *et al.*, 2009).

Biological agents, so-called biocontrol agents, for controlling agricultural pests have been widely used for several years. This is partly due to the strict governmental regulations for the use of hazardous chemicals, as these chemicals can remain protractedly in the food chain (Bro-Rasmussen, 1996). The entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have been found to cause the death of several important agricultural insect pests (Chongchitmate *et al.*, 2005; Grewal *et al.*, 2005). In addition, the bacteria *Xenorhabdus* sp. and *Photorhabdus* sp., living symbiotically in the specialized intestinal vesicles of *Steinernema* and midgut of the intestines of *Heterorhabditis* nematodes, respectively,

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are known to contribute to nematodes' pathogenicity (Gaugler, 2002). After the nematodes (in infective juvenile stage) enter the host insects through natural insect openings, the bacteria are released from nematodes' intestine and enter the host insect haemolymph system. The bacteria then start to replicate rapidly and cause septicemia and cellular apoptosis, leading to death of the host insect (Cho and Kim, 2004; Campos-Herrera *et al.*, 2009).

Both *Xenorhabdus* and *Photorhabdus* bacteria have been shown to grow successfully under laboratory conditions, and both cell suspensions and cell-free supernatants of these bacteria have been found to cause adverse effect and cause the death of several insect pests, such as desert locust [*Schistocerca gregaria* (Forskål)] (Mahar *et al*., 2004), red flour beetle [*Tribolium castaneum* (Herbst)] (Shrestha and Kim, 2010), wax moth [*Galleria mellonella* (L.)], beet armyworm [*Spodoptera exigua* (Hübner)], diamondback moth [*Plutella xylostella* (L.)], cotton leafworm moth [*Spodoptera littoralis* (Boisduval)] (Campos-Herrera *et al*., 2009), and black vine weevil [*Otiorhynchus sulcatus* Germar] (Mahar *et al*., 2008). Hence, these bacteria secrete their metabolic products that are toxic or immunosuppressive to the host insects (Bowen *et al.*, 2000; Sharma *et al.*, 2002; Mahar *et al.*, 2004; 2008; Bode, 2009; Brivio *et al.*, 2010). Moreover, *Xenorhabdus* sp. and *Photorhabdus* sp. have been used for controlling *Luciaphorus* sp., a mushroom mite that has been known to damage several mushroom species (Bussaman *et al.*, 2006; 2009).

Herein, this study was aimed to determine the acaricidal activity of *Xenorhabdus stokiae* isolate PB09 against a mushroom mite (*Luciaphorus* sp.).

2 Materials and methods

2.1 Bacteria, mushroom culture, and mites

X. stokiae isolate PB09 was isolated from surface-sterilized infective juveniles of *Steinernema siamkayai* nematode obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives Thailand, using a method previously described (Kaya and Stock, 1997). A nutrient bromothymol blue triphenyltetrazolium chloride agar (NBTA) medium, consisting of 37 g nutrient agar (Criterion, USA), 25 mg bromothymol blue powder (Lab-Chem, UK), 4 ml of 0.01 g/ml 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, USA), and 1 000 ml distilled water, was used to select the genus *Xenorhabdus* (Lacey, 1997). The bacteria were spread onto NBTA plates, and the plates were sealed and incubated in the dark at 28 °C for 24 h. These bacteria were found to form blue colonies on NBTA agar (indicated Phase I stage) (Stock *et al*., 1998; Grewal *et al*., 2005) and the colonies were selected and sub-cultured to acquire colonies with uniform characteristics. The selected colonies were individually grown in 25 ml of Luria-Bertani (LB) broth (Sigma-Aldrich, USA) and placed in the incubator shaker (200 r/min) at 28 °C for 48 h under complete darkness (Lacey, 1997). The concentration of whole bacterial cell suspension was determined by the plate-count technique (Klement *et al.*, 1990) and adjusted to 10^8 colony-forming units per ml (CFU/ml) using sterile 1 g/L peptone solution. To obtain cell-free supernatant, cell suspension was centrifuged at 2 500×*g* and 4 °C for 5 min and filtered using a 0.22-μm filter. The resulting bacterial pellets were collected and used for extraction of crude cell extract. A total of 10 ml of LB broth was added to 0.1 g (approximately 10^9 cells) of bacterial pellets and then placed in a sonicator at 4 °C for 5 min to break the cells before centrifugation at 2 500×*g* and 4 °C for 5 min, to collect crude cell extract at the top of solution.

Lentinus squarrosulus, obtained from the Mushroom Researchers and Growers Society of Thailand, was grown on a mixture of sawdust and sorghum grain to establish a fresh spawn (Bussaman *et al.*, 2009). Mushroom mycelia were then inoculated to potato dextrose agar (PDA, Sigma-Aldrich, USA) plates and incubated at 25 °C in the dark for further experiments.

Luciaphorus mites were collected from infested *L. squarrosulus* basidiocarps obtained from the Rapeephan mushroom farm in Khon Kaen Province, northeast Thailand. A fresh *L. squarrosulus* spawn in a glass bottle was used to maintain a pair of male and female mites at 28 °C for reproduction. These in-house bred mites were used for all of the experiments.

2.2 Effects of *X. stokiae* isolate PB09 on mortality of *Luciaphorus* sp.

Acaricidal activities of *X. stokiae* isolate PB09 were investigated using 50-mm plastic Petri-dish

plates containing *L. squarrosulus* mycelia grown on PDA, as previously described by Bussaman *et al*. (2009). One hundred adult *Luciaphorus* female mites (1 d old) were transferred to each of these 50-mm plastic Petri-dish plates. A total of 500 μl of bacterial whole cell suspension (1×10^8 CFU/ml), cell-free supernatant, or crude cell extract was then sprayed onto the *L. squarrosulus* mycelia and mushroom mites. The same volumes of LB broth and propargite (a commercial acaricide) at the concentration of 0.04% were used as negative and positive control groups, respectively (four replications/treatment). All plates were covered with lids and placed in a growth chamber at 28 °C and 80% relative humidity in complete darkness. Mite mortality was monitored every 24 h for five consecutive days after treatment. The experiment was repeated twice.

2.3 Effects of *X. stokiae* isolate PB09 on progeny production of *Luciaphorus* sp.

The bacterial cell suspension (1×10^8 CFU/ml), cell-free supernatant, and crude cell extract were tested against *Luciaphorus* mites as described above (four replications/treatment). All plates were covered and placed in a growth chamber at 28 °C and 80% relative humidity in the dark. Five days after the treatment, all living pregnant females were excised using sterile needle and number of eggs/gravid female and the gender of progeny mites were recorded. The experiment was repeated twice.

2.4 Statistical analysis

The data on the percentage of mite mortality were arcsine transformed before analysis. A general linear-model procedure [one-way analysis of variance (ANOVA), SAS Institute, Cary, NC, USA] was used to perform analysis of the treatments. Significant differences between means of the treatment were determined using the least significant difference (LSD) test at $P\leq0.05$.

3 Results

3.1 Effects of *X. stokiae* isolate PB09 on mortality of *Luciaphorus* sp.

Different parts of *X. stokiae* isolate PB09 culture were found to induce mortality of *Luciaphorus* mite at different levels (Table 1). For all the bacterial treatments, the percentages of mite mortality reached a maximum on Day 3 post-treatment and remained unchanged thereafter. Cell-free supernatant of *X. stokiae* isolate PB09 caused the highest mortality of mites up to (89.00±3.60)%, which was not significantly different from that caused by whole cell suspension [(81.66±2.88)%]. Crude cell extract of *X. stokiae* isolate PB09 induced mite mortality [(30.00±5.77)%] to a level significantly lower than cell-free supernatant, whole cell suspension, and propargite. No dead mites were observed after application with LB broth.

3.2 Effects of *X. stokiae* isolate PB09 on progeny production of *Luciaphorus* sp.

The reproduction of *Luciaphorus* mite was found to decline after application with *X. stokiae* isolate PB09 (Table 2). Both cell-free supernatant and whole cell suspension of *X. stokiae* isolate PB09 significantly reduced mite fecundity, accounting for (41.33±23.69) and (192.67±11.01) eggs/female, respectively, when compared to the treatment with LB broth [(256.00±19.69) eggs/female]. Furthermore,

Table 1 Mortality rates of *Luciaphorus* sp. after treated with whole cell suspension, cell-free supernatant, and crude cell extract of *X. stokiae* isolate PB09 for 5 d at 28 °C and 80% relative humidity in complete darkness

Treatment	Mite mortality (%)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Whole cell suspension	42.66 ± 6.80^{cB}	58.33 ± 16.07^{bA}	81.66 ± 2.88^{bA}	81.66 ± 2.88^{bA}	81.66 ± 2.88^{bA}
Cell-free supernatant	55.00 ± 5.00^{bA}	71.66 ± 7.63^{bA}	89.00 ± 3.60^{bA}	89.00 ± 3.60^{bA}	89.00 ± 3.60^{bA}
Crude cell extract	23.00 ± 5.77^{dA}	28.00 ± 0.00^{cA}	30.00 ± 5.77^{cA}	30.00 ± 5.77^{cA}	30.00 ± 5.77^{cA}
Propargite*	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}	100.00 ± 0.00^{aA}
LB broth	0.00 ± 0.00^{eA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{dA}	0.00 ± 0.00^{dA}

Data are expressed as mean±standard deviation (SD). Means within the same column followed by the same lower case letters are not significantly different ($P<0.05$) as compared by LSD test; Means within the same row followed by the same upper case letters are not significantly different ($P<0.05$) as compared by LSD test. * Propargite is a commercial acaricide

Table 2 Progeny of *Luciaphorus* sp. after treated with whole cell suspension, cell-free supernatant, and crude cell extract of *X. stokiae* isolate PB09 for 5 d at 28 °C and 80% relative humidity in complete darkness

Treatment	Fecundity[#] (egg/female)	Egg hatching[#]		Male:female ratio
		Male	Female	
Whole cell suspension	192.67±11.01^{b}	21.00±6.55^{a}	171.66±12.58^{c}	1:8.17
Cell-free supernatant	41.33±23.69^{c}	7.33±2.31^{cd}	34.00±7.54^{d}	1:4.63
Crude cell extract	228.00±23.06^{a}	13.00±3.46^{bc}	215.00±13.23^{b}	1:16.53
Propargite[*]	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{e}	0:0.00
LB broth	256.00±19.69^{a}	15.00±5.00^{ab}	241.00±8.54^{a}	1:16.06

[#] Data are expressed as mean±SD. Means within the same column followed by the same letters are not significantly different (P<0.05) as compared by LSD test. [*] Propargite is a commercial acaricide

cell-free supernatant could reduce the number of eggs/female to a level significantly lower than whole cell suspension. In addition, the male:female ratios of mite progeny showed that the number of female offspring was much reduced by applications with cell-free supernatant and whole cell suspension, accounting for 1:4.63 and 1:8.17 male:female, respectively. In contrast, crude cell extract of *X. stokiae* isolate PB09 showed no effect on mite fecundity [(228.00±23.06) eggs/female] or male:female ratio (1:16.53), similar to LB broth [(256.00±19.69) eggs/female and 1:16.06, respectively]. Also, no mushroom mites were found to survive after propargite application; hence, no fecundity was recorded.

4 Discussion

Different parts of *X. stokiae* isolate PB09 culture, particularly cell-free supernatant and whole cell suspension, demonstrated harmful effects on *Luciaphorus* mortality and fecundity. The mite mortality rate caused by whole cell suspension of *X. stokiae* isolate PB09 in this study was similar to that of *Xenorhabdus* sp. X1 previously published (Bussaman *et al.*, 2009). Also, a decrease of fecundity caused by whole cell suspension of *X. stokiae* isolate PB09 in this study was equivalent to that in a previous report (Bussaman *et al.*, 2009). These results may indicate that whole cell suspension of *X. stokiae* isolate PB09 in the current study is as effective as the previous one. These high mortality rates caused by *X. stokiae* isolate PB09 suggest that *X. stokiae* can transmit horizontally (most likely by direct contact) between infected mites that come in contact with uninfected ones. Moreover, the decrease of fecundity and the changes in sex ratios of mites caused by *X. stokiae* isolate PB09 infection may indicate vertical transmission of *X. stokiae* between pregnant females and their offspring, resulting in sexual bias of their offspring. There are some reports of other microorganisms that have produced similar effects on mites, such as *Microsporidium phytoseiuli* against *Phytoseiulus persimilis* Athias-Henriot and *Wolbachia* bacteria against *Tetranychus urticae* (Koch) (Bjørnson and Keddie, 2001; Vala *et al.*, 2004). However, more experiments are required to verify the effects of *X. stokiae* isolate PB09 on *Luciaphorus* mites.

Cell-free supernatant of *X. stokiae* isolate PB09 in this study was shown to induce both higher mite mortality and lower mite fecundity than the whole cell suspension. This may suggest that metabolites with insecticidal properties that were produced by *X. stokiae* isolate PB09 are more likely to be secreted to culture supernatant. Mahar *et al*. (2005) also found that *X. nematophila* cell-free metabolites required 4 d to kill 95% *G. mellonella* larvae whereas cell suspension needed up to 6 d to induce 93% mortality. There are several reports indicated that *Xenorhabdus* sp. could produce and secrete several secondary metabolites with effective bioactivities such as benzylideneacetone (antibacterial compound), iodinine, phenethylamides, indole derivatives, xenorhabdins, xenorxides, and xenocoumacins (antibiotics), and primary metabolites, such as alkaline protease (Morgan *et al.*, 2001; Caldas *et al.*, 2002; Ji *et al.*, 2004; Mohamed, 2007; Bode, 2009), whereby all of which are suggested to play roles as insecticidal and immunosuppressive compounds. In contrast, crude cell extract of *X. stokiae* isolate PB09 had very little effect on mite mortality or fecundity. This is probably due to the loss of important metabolites of bacterial crude cell extract during the extraction process, or they have simply never been present.

5 Conclusions

In conclusion, different parts of *X. stokiae* isolate PB09 culture produced different effects on *Luciaphorus* mortality and fecundity. As *X. stokiae* cell-free supernatant was shown to be more effective than its whole cell suspension, this may suggest that *X. stokiae* isolate PB09 is more likely to secrete its metabolites with acaricidal activities to the surrounding culture media. This is considered to be important for future formulation of *X. stokiae* isolate PB09 as an environmental- and user-friendly biological control agent. More experiments are required to investigate the appropriate formulas and their effects under field conditions.

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