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In Vitro Bioassay of *Purpureocillium lilacinum* and *Bacillus thuringiensis* for Control of *Meloidogyne incognita* on Black Pepper (*Piper nigrum* L.) in Sarawak, Malaysia, Northern Borneo

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ABSTRACT

This study aimed to evaluate strains of the entomopathogenic fungus *Purpureocillium lilacinum* and the bacterium *Bacillus thuringiensis* from Bintulu, in north-eastern Sarawak, Malaysia for their *in vitro* nematicidal properties against different developmental stages of *Meloidogyne incognita*. Ten indigenous novel strains of *P. lilacinum*, a commercial strain of the fungus (*P. lilacinum* M), and a strain of *B. thuringiensis* carrying Cry6 and Cry14 gene sequences were screened for parasitism against *M. incognita* females and eggs, egg hatching inhabitation and 2nd stage of active juveniles (J2) mortality. Our study demonstrates that *P. lilacinum* A and *P. lilacinum* B were the most effective biocontrol agent against *M.incognita* females, eggs and J2 due to highest infection of females, eggs and significant decrease in egg hatching events. *P. lilacinum* A, *P. lilacinum* B, and *P. lilacinum* M (positive control) demonstrated highly significant infection (>90%, P≤0.01) on *M.incognita* female nematodes. The present study revealed that spore suspension (10^5 spore/mL) of *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M resulted in 78.8%, 66.0% and 73.4% parasitism on eggs, respectively. *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M with low mortality of 6.0%, 5.5% and 5.7%, respectively, showed significant (P<0.05) differences in mortality effect on J2 of *M.incognita* as compared with control treatment (2.3%). Hatching of *M. incognita* eggs incubated in spore suspension of *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M for seven days were reduced by 89% when corrected for control mortality (26%).

Key words: Laboratory bioassay, *Meloidogyne incognita*, *Purpureocillium lilacinum, Bacillus thuringiensis*, biocontrol, root-knot nematodes

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INTRODUCTION

Black pepper (*Piper nigrum* L.) (Piperales: Piperaceae), the king of spices, is an important cash crops supporting the livelihood of about 67,000 rural dwellers in upland areas of Sarawak, Malaysia in northern Borneo. Such areas are loosely defined as generally remote, interior, hilly to mountainous landscapes and tablelands at moderate to high elevations where dryland farming is dominant (Li, 1999). Sarawak has the right latitude, sufficient level of rainfall, and suitable soil profile and topography for planting quality pepper. The black pepper industry is the largest agricultural export commodity of Sarawak, with production area of approximately 16,021 hectares producing 34,294 tonnes of peppercorn (MPIC, 2013). Annual export income was valued at RM 1.95 billion or USD 0.48 billion in 2019. *Piper nigrum* is attacked by several pests and diseases (Kueh,1986), the most economically important of which in Sarawak are root-knot nematodes (RKN), *Meloidogyne* spp. (Heteroderidae), that threaten the viability and sustainability of the black pepper industry (Kueh & Teo, 1978; 1990; Leong, 1986; Kueh & Sim, 1992; Ramana & Eapen, 2000; Eng, 2001).

In the absence of effective control of RKN, badly infested vines are stunted with some yellowing and gall formation on the roots. Vines subsequently become unproductive and are abandoned, resulting in substantial economic losses to pepper farmers. Currently, no RKN resistant black pepper cultivar is available in Sarawak (Eng, 2001) and nematicides are usually expensive and may raise problems of environmental pollution and/or of accumulation of toxic residues in edible plant products. They also require frequent application to be effective against nematodes (Gowen,1997). On the other hand, microbial control agents (MCAs) are gaining popularity in integrated nematode management programs due to promising results of their use and relative safety with respect to synthetic nematicides (Mukhtar & Pervaz, 2003; Dong & Zhang, 2006).

Purpureocillium lilacinum ((Thom) Luangsaard et al (2011)) (*Ophiocordycipitaceae*), a saprophytic soil fungus known as a natural facultative egg parasite of root-knot and cyst nematodes (Kannan & Veeravel, 2012) has gained attention of the researchers over the past decade due to its efficacy as a parasite for suppressing populations of phytophagous nematodes (Jatala, 1986; Dube & Smart, 1987; Freitas, Ferraz, & Muchovej, 1995; Khan et al, 2006a; Kiewnick & Sikora, 2006; Oclarit & Cumagun, 2009; Brand, Soccol, Sabu, & Roussos, 2010; Kannan & Veeravel, 2012; Timper, 2014). It was reported with high frequency of occurrence in the tropics and subtropics (Morgan-Jones, White, & Rodriguez-Kabana, 1984; Akyazi & Dickson, 2014) and can be found in most of agricultural soils (Brand et al., 2010). Eng (2001) reported that 82.9% of 43 surveyed farms in Sarawak contained *P. lilacinum* despite intensive application of nematicides in the farms. Cabanillas et al. (1989) observed maximum growth of *P. lilacinum* at temperature ranged from 24ºC-30ºC. They reported that *P. lilacinum* was able to grow on a wide range of common organic substrates and remain competitive with other microbes in the soil. It also tolerated broad range of soil pH and was able to grow well at 15°-30°C. Since *P. lilacinum* has high adaptability in its life strategy, it is competitive in a broad spectrum of range adaptability.

Bacillus thuringiensis (Bt) Berliner (*Bacillaceae*), a well-known entomopathogenic bacterium used for the control of insects for more than four decades, (Brar, Verma, Tyagi, Valéro, & Surampalli, 2006) has also drawn intensive studies on its nematicidal effects against economically important phytophagous nematodes (Devidas & Rehberger, 1992; Siddiqui & Mahmood, 1994; Carneiro, Souza, & Belarmino, 1998; Mozgovaya, Byzov, Ryabchenko, Romanenko, & Zvyagintsev, 2002; El-Nagdi & Youssef, 2004; Mohammed et al, 2008; Khan et al, 2010). Carneiro et al (1998) claimed that *B. thuringiensis* was efficacious in killing freshly hatched 2nd stage juveniles (J2) of sugarcane eelworm, *Meloidogyne javanica* (Treub,1885) Chitwood, 1949. Mozgovaya et al (2002) reported 80% mortality of nematodes after *in vitro* treatment with *B. thuringiensis*. El-Nagdi & Youssef (2004) found that soaking faba beans with *B. thuringiensis* reduced the population density of *M. incognita* and increased the plant growth. According to Osman, Ameen, Mohamed, El-Mohamedy, & Elkelany (2018), *B. thuringiensis* applied alone improved the growth parameters of eggplant and reduced nematode development. Mohammed et al (2008) reported that the spore/crystal proteins of *B. thuringiensis* showed high nematicidal activity against *M. incognita* (Kofoid and White) Chitwood. However, no current report was found on the positive effect of *B. thuringiensis* in controlling RKN development in black pepper. Therefore, studies were conducted to investigate the feasibility for use of *P. lilacinum* and *B. thuringiensis* as alternatives to the chemical nematicides currently used to control RKN in black pepper. The objective of this study was to evaluate the local virulent strains of *P. lilacinum* and *B. thuringiensis* for their *in vitro* nematicidal properties against different developmental stages of *M. incognita* in the laboratory bioassay.

MATERIAL AND METHODS

Establishment of pure *Meloidogyne incognita* (MI) culture

A pure population of *M. incognita* was established on a local variety of tomato (*Solanum lycopersicum* L. (Solanales: Solanaceae) from a single egg mass of *M. incognita*, and maintained on tomato plants raised in pots filled with sterilized pot mixture (2: 1: 1 sand, loam, cocoa peat, respectively).

Isolation, screening, and identification of *Purpureocillium lilacinum* (PL)

Seven indigenous strains of *P. lilacinum*, designed as *P. lilacinum* A, *P. lilacinum* EJ1, *P. lilacinum* EJ2, *P. lilacinum* EK1, *P. lilacinum* EK2, *P. lilacinum* EK3, and *P. lilacinum* EK4 were isolated from egg masses and females of *M.incognita* while three indigenous strains, designed as *P. lilacinum* 1A, *P. lilacinum* B and *P. lilacinum* SA were obtained from soil. *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M were further confirmed at molecular level with primer pairs: ITS1-ITS4 (Inglis & Tigano, 2006) and EF4-EF3 (Glass & Donaldson, 1995) before being assessed for their *in vitro* antagonism on egg masses, eggs, egg hatch and 2nd stage of juveniles (J2) of *M. incognita*. The sequences for ITSI, ITS4 and EF3, EF4 genes were listed as follows:

ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'),

ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and

EF3 (5′-TCCTCTAAATGACCAAGTTTG-3′),

EF4 (5′-GGAAGGG[G/A]TGTATTTATTAG-3′).

Identification of *P. lilacinum* was based on classical (morphological and morphometric properties) and molecular methods (Luangsa-Ard et al, 2011). *P. lilacinum* M, a commercial strain (SUBOTANI, Malaysia) served as positive control in all tests. Roots and rhizosphere soils around black pepper roots were collected from several areas in two black pepper farms, one at Bintulu (3.2167°N, 113.0989°E) in north-eastern Sarawak and the other at Kuching (1.4497°N, 110.1339°E) in the south-eastern Sarawak, where RKN disease is prevalent. Root pieces were washed in gentle running tap water for 5 min. Females and egg masses were extracted from roots using method described by Sun, Gao, Shi, Li, & Liu (2006) before transferred to PDA+ [Potato Dextrose Agar (Merck, Darmstadt, Germany) amended with 0.01%(w/w) chloramphenicol (Sigma, USA) and 3% (w/w) sodium chloride] plate. To isolate from soil: Serial dilution and a standard pour plate technique were used (Thomas, Sekhar, Upreti, Mujawar, & Pasha, 2015). Dilution at 10^{-1}, 10^{-2} and 10^{-3}, were subsequently spread onto PDA+ plates and incubated at room temperature (28° ± 1°C) for 7 days.

Determination of the effect of *P. lilacinum* on female nematodes of *M. incognita*

P. lilacinum A, *P. lilacinum* EJ1, *P. lilacinum* EJ2, *P. lilacinum* EK1, *P. lilacinum* EK2, *P. lilacinum* EK3, *P. lilacinum* EK4, *P. lilacinum* 1A, *P. lilacinum* B, *P. lilacinum* SA and *P. lilacinum* M were preliminarily screened for their efficacies in infecting *M.incognita* female nematodes. Five female nematodes of approximately equal size (from pure culture) were surface-sterilized with 1.0% sodium hypochlorite for 5 minutes after extracted from tomato roots. These were later collected on a 200-µm sieve and were rinsed 3 times with chlorine-free sterile tap water (Sun et al*.,* 2006) before placing at the edge of 5-day-old fungal colony grown on water agar 2.0 % (w/w). Plates were arranged according to a Simple Randomized Design (SRD), run in four replicates and incubated at room temperature (28°± 2 °C). After 4 days, females were observed under stereo microscope (45x) to detect emerging mycelium from the body surface as sign of colonization.

P. lilacinum* (PL) infection rate on females and egg masses of *M.incognita

Infection rate on *M.incognita* females by selected *P. lilacinum* was conducted with ten females per plate and four replications per fungus treatment. Sign of colonization was observed daily until the 6th day. Similarly, the procedure was repeated for egg masses.

Infection on eggs of *M. incognita*

M.incognita egg suspensions were prepared as described by Nitao, Meyer, & Chitwood (1999). Fifty (50) µL freshly prepared egg suspensions (approximately 300 fresh eggs) was pipetted separately into sterile McCartney bottles and to which

1 mL of fungus spore suspension (10^5 spores/mL) prepared in sterile distilled water was added. Sterile water was added for control treatment. The entire procedure was conducted in aseptic condition. Bottles were sealed with Parafilm (Sigma, USA), arranged in a CRD and placed in the dark at room temperature (28° ± 2°C) with four replications per fungus treatment. Four days later, drops of egg suspension from each replicate (approximately 100 eggs) were pipetted onto glass slides, stained with lactophenol cotton blue and examined under a compound microscope (×400) for signs of parasitism.

Impacts of spore suspension of *P. lilacinum* on egg hatch and 2nd stage juveniles (J2) of *M. incognita*

Procedures described for egg parasitism test were repeated with fresh material but the incubation period was extended to seven days. Egg hatch rate and juvenile stage 2 (J2) mortality were determined by counting all eggs, J2 and dead J2 in a counting disc under a stereo-microscope, which were later calculated to determine the percentage of egg hatch and percentage of J2 mortality according to the following formula:

Egg hatch percentage = 100 x J2/(eggs + J2)

J2 mortality percentage = 100 × dead J2/total J2,

A piece of eyelash attached to a tooth- pick was used to probe the tail of J2. They were considered dead if they became rigid and did not react when probed by the eyelash.

Isolation of *Bacillus thuringiensis* (Bt)

Soil samples were randomly collected from several locations of a cattle farm at the University Putra Malaysia (UPM) Bintulu Sarawak Campus (3.2058°N, 113.0999°E). Sodium acetate- (0.25 M) -selection heat-pasteurization, and 50% ethanol treatment methods were used for *B. thuringiensis* isolation as described by Xavier, Reena Josephine, & Sreeramanan (2007). *B. thuringiensis* colonies were selected based on *B. thuringiensis* colony morphology, with the appearance of a fried egg on the plates (Barathi, Sangeetha, Karthick, Govindaraju, & Indra Arulselvi, 2012). *B. thuringiensis* was again inoculated in nutrient broth medium for sporulation. The culture was further examined and confirmed under phase contrast microscope.

The production of parasporal crystal (a solid crystalline protein which is produced next to the endospore during spore formation of *B. thuringiensis*) in *B. thuringiensis* culture was detected by adopting crystal protein staining method of Sharif & Alaeddinoğlu (1998). Smear of 2-day-old culture of *B. thuringiensis* prepared on a glass slide was dipped in a Coomassie brilliant blue solution (0.25% Coomassie brilliant blue, 50% ethanol and 7% acetic acid) for 3 minutes, then washed with tap water, dried and observed under a light compound microscope at 1000x magnificent without cover and oil immersion.

Detection of Cry Gene with Molecular Method and forward

DNA of an overnight *B. thuringiensis* culture was extracted according to DNA extraction procedure for *P. lilacinum* except incubation of pellet in 180 µL of TE buffer at 100°C for 10 minutes was omitted. Forward and backward primers of Cry6 and Cry14 (Salehi Jouzani et al, 2008) were performed, replacing ITS and EF primers, in order to detect nematode-specific Cry active genes, Cry6 and Cry14. The sequences for Cry6 and Cry14 genes were as follows: Cry6+F (5'-TGG CGT AGA GGC TGT TCA AGT A-3'), Cry6-R (5'-TGT CGA GTT CAT CAT TAG CAG TGT-3') and Cry14+F (5'-ATA ATG CGC GAC CTA CTG TTG T-3'), Cry14-F (5'-TGC CGT TAT CGC CGT TAT T-3').

Bioassay on the toxicity of *B. thuringiensis* parasporal crystal on 2nd stage juveniles (J2) of *M. incognita*

A bioassay method developed by Zi-Quan et al (2008) was modified and used for testing the parasporal crystal protein of *B. thuringiensis* against *M. incognita*. *B. thuringiensis* isolate that carry Cry active genes (Cry6 and Cry14) was selected for this study and named as *B. thuringiensis* Bt 614. The presence of Cry6 and Cry14 genes in *B. thuringiensis* served as an indication for the production of parasporal crystal 6 and crystal 14, which can be toxic and show activity against root-knot nematodes. The selected *B. thuringiensis* 614 initially was grown in nutrient broth on a rotary shaker (180 rotations per minute) for 2 days at 32°C in order to reach stationary phase with cell density of (1-5) x 10^9 CFU/mL (Mozgovoya et al, 2002). *B. thuringiensis* broth culture was subjected to centrifugation at 12,000 g for 20 minutes for precipitation of cells, spores and crystals (Carneiro et al, 1998). The precipitates were washed two times in chilled distilled water by centrifugation before re-suspended in sterile distilled water to the initial volume.

A suspension (1.85 x 10^4 CFUs) of *B. thuringiensis* spore, crystals and cells were two-fold serially diluted to achieve concentrations of 50%, 25%, 12.5% and 6.25%. Two mL of each concentration was added to 3 mm diameter glass dish. Two mL of J2 *M. incognita* suspension (average 62 *M. incognita* juveniles/mL) was later added into each dish. Glass dish containing 2 mL of sterile tap water and 2 mL of J2 suspension served as control. There were four replications for each treatment, run in a CRD. After 24, 48 and 77 hours of incubation periods, each dish was stirred for 30 seconds to mix the content before placed under a dissecting microscope to determine the number of paralyzed J2 from a total count of 50 nematodes. Mean percentage of paralyzed J2 was estimated.

Statistical analysis

Means of data in percentage subjected to arc-sine transformation, were analysed according to standard procedure for analysis of variance (ANOVA). Differences between means were compared using SAS version 9.0 for significance according to Duncan multiple range test ($P<0.01$ and $P<0.05$). Untransformed arithmetic means are reported.

RESULTS AND DISCUSSION

Determination of the impact of *P. lilacinum* on female nematodes of *M. incognita*

Fig. 1 showed that infection of *M. incognita* females by all *P. lilacinum* isolates differed according to the isolate, with isolate *P. lilacinum* Ek2, *P. lilacinum* Ek4 and *P. lilacinum* EJ2 causing lower infection levels and *P. lilacinum* 1A, *P. lilacinum* EK1, *P. lilacinum* EK3 in moderate infection levels while *P. lilacinum* A, *P. lilacinum* M, *P. lilacinum* B, *P. lilacinum* EJ1 and *P. lilacinum* SA resulting in highly significant (P<0.0001) infection levels (>80%). Similar results were reported by Eapen, Beena, & Ramana (2005) on the significant infection of *P. lilacinum* isolate on *M. incognita* females. A stereo microscope (40×) observation demonstrated radiating mycelia from the female's body (Fig. 2a and b) unrestricted to vulva, anus or broken opening, which corroborates that appressoria are not involved in the penetrating process due to the lack of a tough cross-linked chitin layer on the female body as described in early studies (Morgan-Jones et al,1984; Holland, Williams, & Khan, 1999; Khan et al,2006b). However, according to Jatala (1986), Peruvian isolate of *P. lilacinum* infected the female of *M. javanica* (Treub) only by body opening. Khan et al (2006b) suggested that this penetrating process may be attributed to different profile enzymes of each strain that help in the direct cuticle penetration.

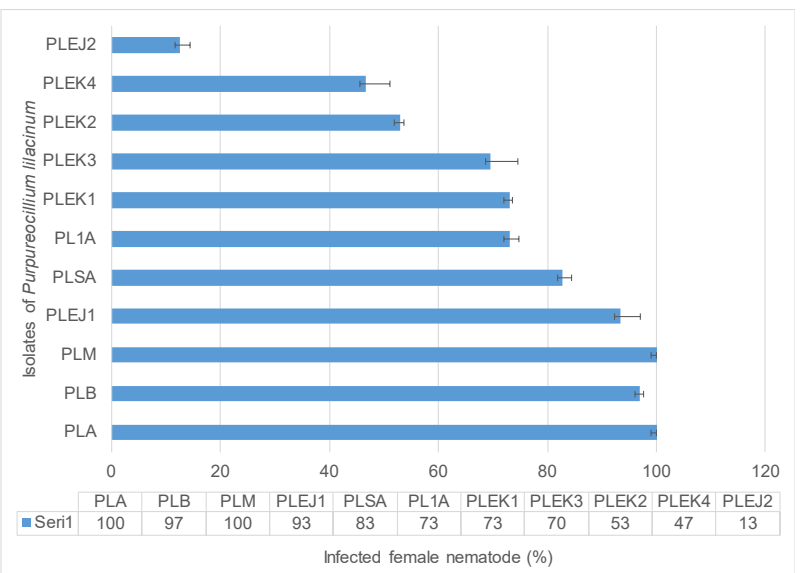

Fig. 1 Percentage of female Meloidogyne incognita infection by *Purpureocillium lilacinum*, determined by observing emerging hyphae from the body surface after 4 days of incubation under a stereo microscope at 40×. Value represents mean (%) of five replications. Means followed by the same letter are not significantly different according to Duncan Multiple Range Test at P < 0.01. Vertical bars indicate standard error of the means.

P. lilacinum infection rate on females and egg masses of *M. incognita*

Fig. 2a demonstrated higher rates of infection on *M. incognita* females by isolates *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M. Both *P. lilacinum* A and *P. lilacinum* M on the 1st day reached 50% infection rate followed by *P. lilacinum* B with a rate of 10%. Nevertheless, all isolates eventually achieved 100% infection on the 6th day. It was observed that from the 3rd day onwards, there was no significant increase in percentage of infection on female nematodes by the three above isolates.

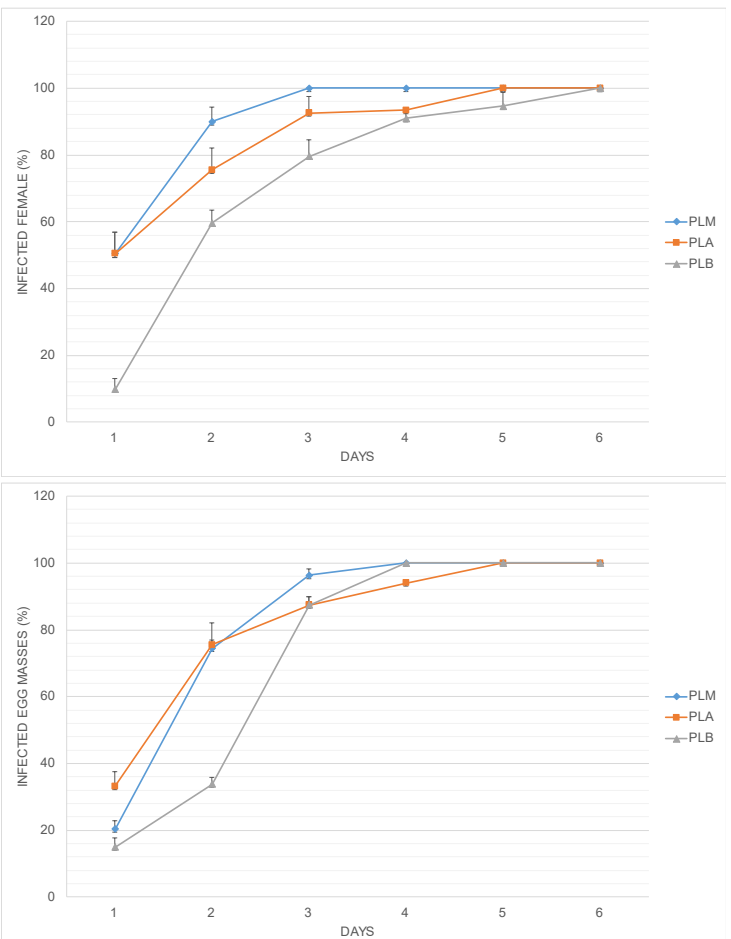

Fig. 2 Infection rate of *Purpureocillium lilacinum* on *Meloidogyne incognita* (a) infected female nematodes and (b) infected egg masses under in vitro condition. Sign of infection by observing the emerging hyphae from the surface of specimens (female or egg mass). Each value represents the mean [a % of infected female; b % of infected egg masses] of 5 replications (n=5). Means followed by the same letter for each respective day are not significantly different according to Duncan Multiple Range Test at $P < 0.05$. Vertical bars indicate standard error of the means.

The infection of egg masses by *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M was confirmed by observing the emerging mycelium from the surface of egg masses under a stereomicroscope 40× (Figs. 3a, 3b and 3c). On the first day, the rates of infection of egg masses ranging from 15 to 38% were recorded by *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M and then it increased to 90-95% on the third day and eventually achieved complete infection on the fifth day (Fig. 2b). There was no significant increase in percentage of infection of egg mass among *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M from the 3rd day onwards. This was due to the presence of antimicrobial compound (as suggested by Orion, Kritzman, Meyer, Erbe, & Chitwood, 2001) in gelatinous matrix (GM) of an egg mass can protect eggs from microbial infection (Kannan & Veeravel, 2012). An effective parasite generally should be able to utilize GM as source of nutrient and then reproduce in it (Sharon et al, 2007). Since the mycelium was detected on the surface of GM in the present study, suggesting that *P. lilacinum* A and *P. lilacinum* B possess resistance towards antimicrobial compound present in the GM. This observation is in agreement with the studies on *Meloidogyne* spp. by Zaki & Batti (1991) and Eapen et al (2005).

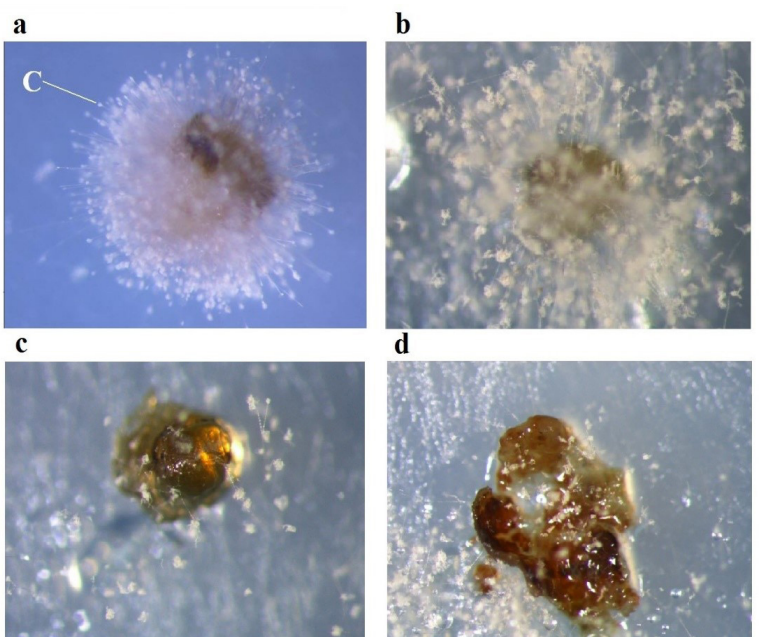

Fig. 3. Infection on *Meloidogyne incognita* female by PLM (a) & PLA (b); hundreds of conidiosphores (C) radiating from the body surface. c and d Infection on egg mass by PLA; growing mycelium was detected on the surface of egg mass (c and d). Sign of infection was detected by observing the emerging hyphae from the surface of specimens (female or egg mass) under a stereo microscope at 40×.

P. lilacinum parasitism on eggs of *M. incognita*

The results demonstrated significant (P<0.05) parasitic effect on *M. incognita* eggs with 78.5%, 73.4% and 66.0% parasitism for *P. lilacinum* A, *P. lilacinum* M and *P. lilacinum* B as compared with the control. However, it was noted that they did not differ significantly in percentage parasitism among themselves (Table 1). This result confirmed the observation made by Sun et al (2006) who reported a high *in vitro* parasitism rate of *P. lilacinum* strain YES-X-2-14 on *M. hapla* Chitwood eggs. In addition, another thirty *P. lilacinum* isolates of Sun et al (2006) were reported to parasitized 100% of *M. hapla* eggs. Al Kader (2008) stated that 77% infection of *M. incognita* eggs after 4 days of incubation.

Table 1. Impact of PL spore suspension (10^5 spores/ml) on parasitism of Meloidogyne incognita eggs, egg hatch inhibition and J2 mortality.

Isolate	Parasitized egg (%)	Hatch Inhibited egg (%)	J2 Mortality (%)
PLA	78.5 ± 2.4 [a]	88.3 ± 1.4 [a]	6.0% ± 1.8 [a]
PLB	66.0 ± 5.2 [b]	88.2 ± 1.7 [a]	5.5% ± 0.8 [a]
PLM	73.4 ± 0.6 [ab]	89.4 ± 1.0 [a]	5.7% ± 1.0 [a]
Control	0.0 ± 0 [c]	25.9 ± 3.12 [b]	2.3% ± 0.8 [b]

Each value (%) represents the mean of four replications ± standard error. Means within each column followed by the same letter are not significantly different according to Duncan Multiple Range Test at P<0.05.

This study revealed that the eggs in their early stage with no sign of apparent juvenile shape being detected in the embryo were more susceptible to *P. lilacinum* A and *P. lilacinum* B infection than eggs at a later stage in which the embryo was already developed into an identifiable juvenile shape (Fig. 4b and 4c). In most cases, none of the juvenile 1 (J1) was detected in the infected eggs and the embryos seemed to be disintegrated (Fig. 4a, 4d and 4e), which reflected a parasitic effect of *P. lilacinum* on the developing embryos. In several eggs containing developing J2, emerging mycelium was also detected on the surface of these eggs and J1 appeared motionless. This has suggested that both *P. lilacinum* A and *P. lilacinum* B parasitized not only the immature eggs but also some mature eggs containing developing J1. Similar observations were made in the early studies on parasitized eggs of *Meloidogyne* spp. by Morgan-Jones et al. (1984), Irving & Kerry (1986), Jatala (1986), Lopez-Llorca & Duncan (1991), Holland et al (1999) and Eapen et al (2005). This attribute offers advantage in biological control since eggs of all *Meloidogyne* spp. are the major target of plant-parasitic fungus and can remain dormant in the soil for long periods of time.

Under microscopic observation, the hyphae of *P. lilacinum* form an extensive network were ramifying several eggs (Fig. 4f) but not growing on other adjacent eggs in the group. A simple, swollen, hyphal structure recognized as appressorium (Fig. 4f, AP) appeared at the end of hyphae is likely to be in contact with eggshells. Some incubated eggs appeared to be abnormal, deformed and shrunken may be due to the pressure exerted by the network of hyphae. The use of mechanical means to penetrate host was also reported by Holland et al (1999).

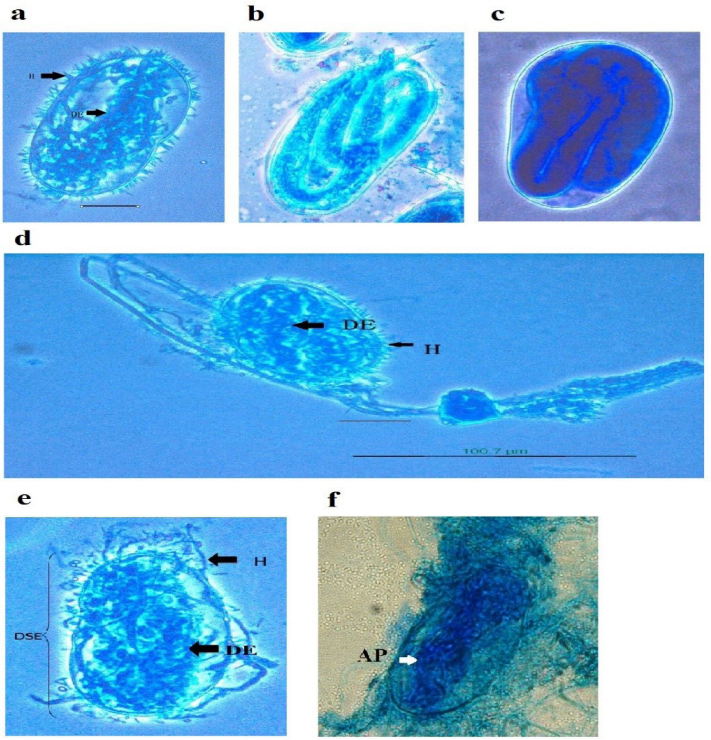

Fig. 4. Parasitism of *Purpureocillium lilacinum* on *Meloidogyne incognita* eggs. Hyphae (H) emerged from a deformed shape egg (DSE) with disintegrated embryo (DE) of *Meloidogyne incognita* after 4 days of incubation. It also penetrated into the egg and consumed the egg content (400x magnification, photo a, d, e & f). Photo b & c: uninfected egg in control treatment. Photo f: Appresorium (AP) pressing on the egg. ('–' is correspond to actual distance of 25 µm).

Once an egg is infected, the nutrient in the egg induces proliferation of hyphae on the egg, for enabling growth to adjacent eggs. In fact, eggshells of nematodes are the barriers to fungus infection (Morton, Hirsch, & Kerry, 2004). They consist of three layers, namely a vitelline layer predominantly composed of proteins, a chitin-protein complex as the middle layer, which is responsible for egg structural strength (Gortari & Hours, 2008) and lastly, an inner lipo-protein layer that protects egg from harmful chemical but loses its protective effect once the chitin layer is destroyed. According to Lopez-llorca, Olivares-Bernabeu, Salinas, Jansson, & Kolattukudy, (2002), Lopez-llorca, MaciÁ-Civente, & Jansson (2008), when a hypha encounters an egg surface, it forms appressoria (Fig. 4f) that subsequently adhere (using extracellular material on appressoria) to the egg for better binding of the fungus to the host (Lopez-llorca et al, 2008). From these appressoria, the fungus uses enzymatic and mechanical means to penetrate the host (Huang, Zhao, & Zhang, 2004; Gortari & Hours, 2008; Lopez-Llorca et al, 2008). The secretions of chitinase and proteases by *P. lilacinum* facilitate egg penetration by breaking down layers in eggshells (the barrier)

so that a narrow infection peg can push through (Morton et al, 2004). The combined effect of chitinase and protease produced by *P. lilacinum* in degrading eggshell layers of *M. javanica* was reported by Khan et al (2004). The lipid layer disappeared while the chitin layer was much reduced after enzyme treatment. Besides, the involvement of serine protease, an extracellular protease penetrating the eggshell has been reported by Mérillon & Ramawat (2012). This enzyme degrades vitellin component in immature eggs. They claimed that addition of chitin or vitellin to the fungal culture medium may induce proteolytic enzymes.

Impact of *P. lilacinum* on egg hatching of *M. incognita*

There are no significant differences in egg hatching inhibition rates (percentages) between all three *P. lilacinum* isolates (*P. lilacinum* M, *P. lilacinum* A and *P. lilacinum* B) with 89.4± 1.0%, 88.3± 1.4% and 88.2± 1.7%, respectively, although significant ($P<0.05$) differences were observed among the treatment and the inoculated control (Table 1). Most eggs in the control treatment appeared empty, with the presence of many J2, suggesting hatching of eggs had occurred whereas eggs treated with spore suspension appeared to be shrunken, deformed and with multiple vacuoles. These studies corroborate the findings by Costa, Campos, Pfenning, & Oliveira (2001) & Sun et al (2006) who reported that culture filtrate of *P. lilacinum* grown in Czapek broth greatly reduced egg hatching of *M. incognita* and average 58% egg hatch inhibition of *M. incognita* for their 186 *P. lilacinum* isolates and further confirm the higher rate of parasitized eggs and egg hatch inhibition of *M. incognita.* According to Mérillon & Ramawat (2012), hatching of eggs containing mature juveniles appeared to be stimulated when incubated in culture filtrate of *P. lilacinum* but development of immature eggs appeared to be disrupted. Under some conditions, egg hatch percentage of plant-parasitic nematode was reported by Chen, Dickson, & Mitchell (2000) to be higher in diluted culture filtrates of some fungi than in the media itself. They suggested that a hatching stimulant in the culture filtrate released by the fungi may gradually reduce the concentration of hatching inhibitor in the media by consuming the nutrient that contribute to a higher egg hatch percentage. This suggest that experimental conditions, fungal strains, culture media, and nematode species are among the different factors which may contribute to contradictory results.

Impact of *P. lilacinum* on mortality of 2nd stage juveniles (J2) of *M. incognita*

In this study, no significant differences in mortality effect on J2 of *M. incognita* between all three *P. lilacinum* isolates (*P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M) with low mortality of 6.0%, 5.5% and 5.7% respectively, although significant ($P<0.05$) differences were observed among the treatment and the inoculated control (2.3%) (Table 1). The results of present study confirmed the findings of Sun et al (2006) who reported a low average J2 mortality percentage of 16% for their 186 *P. lilacinum* isolates. Furthermore, it is in agreement with the reports by other researchers such as Jatala (1986), Bonant et al (1995), Singh & Mathur (2010) that *P. lilacinum* primarily parasitized eggs but not juveniles of *M. incognita*. However, *P. lilacinum*

strain YES-X-2-14 was reported in exhibiting high *in vitro* nematicidal effect on J2 of *Meloidogyne* spp. (Sun et al, 2006). Similarly, Al Kader (2008) reported a high nematicidal effect of their *P. lilacinum* culture filtrate on J2 of *M. incognita*, with 99% of J2 immobilized after 2 days of treatment. It is suggested that different strain of *P. lilacinum* can exhibit different nematicidal effect on J2. Throughout the years, metabolites in culture filtrate of *P. lilacinum* have been detected and screened for paecilotoxin (Singh, Pandey, & Goswami, 2013), acetic acid (Favre-Bonvin, Ponchet, Djian, Arpin, & Pijarowski, 1991), and leucinostatins (Park et al, 2004). These metabolites may potentially result in death on J2 of *M. incognita*. The low mortality effect on J2 of *M. incognita* by spore suspension of the three *P. lilacinum* isolates (namely *P. lilacinum* A, *P. lilacinum* B and *P. lilacinum* M) in this study might be attributed to the absence of digestive enzyme secreted by the *P. lilacinum* or the amount of secreted enzyme was far below the threshold level to invade and penetrate the cuticle of J2 individuals. To infect J2 of *M. incognita*, *P. lilacinum* firstly needs to overcome the cuticle of nematode which is a non-cellular layer production of the hypodermis consisting of keratin, collagens and fibers (Huang et al, 2004). Once the cuticle is penetrated by fungal hyphae, the plant-parasitic nematodes *M. incognita* are then paralyzed, invaded and digested (Soares Sufiate, & de Queiroz, 2018).

Bacillus thuringiensis (Bt)'s parasporal crystal toxicity on 2nd stage juveniles (J2) of *M. incognita*

After 24 hours incubation, none of the treatments (3.12%-50.00% of harvested *B. thuringiensis* toxin, which includes cells, spores and crystals) affected the mobility of J2 (Table 2). *B. thuringiensis* crystal and endospores were ineffective in killing the J2 of *M. incognita*. The observation was continued for 48 hours and 77 hours, however, *B. thuringiensis* crystal proteins also did not demonstrate contact nematicidal effects on J2 of *M. incognita* for both periods of incubation. There was no significant difference on the percentage of paralyzed J2 among the treatments, which is in agreement with the finding of Devides & Rehberger (1992) that purified *B. thuringiensis* toxin did not manifest contact nematicidal activity toward J2 of *Meloidogyne* spp. but was 100% active against the free-living nematode, *Caenorhabditis elegans* (Maupas) (Rhabditidae). There are several reasons that explain the ineffectiveness of harvested parasporal crystals of *B. thuringiensis* in killing J2 of *Meloidogyne* spp. First, phytonematodes including *M. incognita* have a modified feeding structure (known as stylet) which is too small to engulf material actively from soil as compared to bacteriophagous nematodes and substrate ingestor nematode. Therefore, the low mortality effect of *B. thuringiensis* on J2 of *M. incognita* may be due to the inability of its stylets to ingest *B. thuringiensis* toxin (crystal protein) as reported by Mozgovaya et al (2002) and Yu et al (2008). In fact, the presence of specific crystal protein genes in *B. thuringiensis* does not guarantee its toxicity because the genes may not be actively expressed or expressed in a concentration below the threshold level or under the control of a promoter which is not efficient (as cited by Salehi Jouzani,

2008; Jansson et al, 1997; Ferrandis, Jua'rez-Pe'rez, Frutos, Bel, & Ferré, 1999). Moreover, the toxicity of *B. thuringiensis* towards plant-parasitic nematodes can also be affected by solubilization and activation of crystal proteins before ingestion by pests (as cited by van Frankenhuyzen, 2009). Without an alkaline pH, a crystal protein will not be able to dissolve in the midguts of nematodes, thus losing its toxicity effect on J2. Even though ingested crystal protein may show a certain level of toxicity, it can be proteolytically unstable after ingestion and thus not induce J2 mortality. Moreover, in the crystal proteins bioassay of Salehi Jouzani (2008), solubilization of *B. thuringiensis* crystal with 10 mmol/L of mercaptoethanol for 4 h at 37°C was included as one of the steps prior bioassay. In the present study, the technique of Carneiro et al (1998) was adopted without incorporating *B. thuringiensis* crystal solubilization as one of the steps before bioassay. It is speculated that the insolubility of *B. thuringiensis* crystal may also have contributed to its impact on J2 mortality in this study with *M. incognita* [although undiluted *B. thuringiensis* crystal (100% from crude harvest) was used in the bioassay]. Therefore, it is suggested that crystal protein solubilization should be included as an important step in all bioassays related to *B. thuringiensis* and *M. incognita*.

Table 2. Percentage of paralyzed J2 of *Meloidogyne incognita* after 24, 48 and 77 hours exposed to extract of spore-crystal mixtures of *B. thuringiensis*.

BT	24 Hours	48 Hours	77 Hours
50%	5.6 ± 1.0	13.0 ± 2.6	3.5 ± 1.0
20%	4.5 ± 1.5	6.0 ± 1.4	5.5 ± 0.5
12.5%	6.0 ± 1.4	5.6 ± 1.7	4.0 ± 0.3
6.25%	4.4 ± 0.7	4.4 ± 1.5	4.0 ± 1.2
3.12%	5.6 ± 1.5	4.0 ± 1.4	3.5 ± 1.0
Control	3.6 ± 1.2	3.6 ± 1.5	4.0 ± 0.0

Each value represents the mean (%) of five replicates of paralyzed J2± standard error, in the same column are not significantly different according to Duncan Multiple Range Test at $P < 0.05$, n=5.

In contrast to the above finding, Prasad, Tilsk, & Gollakota (1972) claimed that *B. thuringiensis* toxin at 10- fold dilution of fermentation beer, caused total mortality of J2 of *M. incognita* within 24 hours of incubation. Besides, Khan et al (2010) reported a 50% concentration of *B. thuringiensis* cell free culture filtrate significantly ($P<0.001$) increased mortality of J2 in their *in vitro* studies. Studies by other researchers have revealed that the toxicity of *B. thuringiensis* towards nematodes is due to the extracellular β-exotoxins produced in the supernatant of the culture medium (Carneiro et al, 1998; Palma, Muñoz, Berry, Murillo, & Caballero, 2014). Carneiro et al (1998) reported that sporulated cells and δ-endotoxin of *B. thuringiensis* had no nematicidal effect on J2 of *M. javanica*. Surprisingly, Mohammed et al (2008) reported that the spore/crystal proteins of two *B. thuringiensis* isolates (*Bt7N* and *BtDen*) induced 100% mortality on J2 of *M. incognita*. Also, crystal protein toxin of *B. thuringiensis* strain YBT-021 was claimed manifesting toxicity effect to phytonematodes: *M.*

hapla, Pratylenchus scribneri Steiner in Sherbakoff & Stanley (Pratylenchidae), *Tylenchorhynchus* sp. (Belonolaimidae), potato tuber nematode (*Ditylenchus destructor* Thorne (Anguinidae), and *Aphelenchoides* sp. (Aphelenchoididae) (Yu et al, 2008). Furthermore, Khyami-Horani et al (2003) revealed the toxicity of parasporal crystal protein of *B. thuringiensis* towards 3rd stage larvae of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), J2 of *M. javanica* and *M. incognita*. When *Meloidogyne* spp. was treated with fluorescent labelled crystal protein toxin, the fluorescent signal accumulated in the intestinal tissue was detected (Yu et al, 2008). However, no study has been established in the entry mechanism of crystal protein into the intestinal tissue (Yu et al, 2008). Salehi Jouzani et al (2008) reported that newly hatched J2 were not affected by the parasporal crystal proteins of *B. thuringiensis* isolates YD5 and KON4 at 2 x 10^8 CFU/mL concentration after 24 hours of incubation. However, after 3-4 days of incubation, egg hatching inhibition was detected at 46% and 45% respectively, besides manifesting mortality effect on *M. incognita* at 77% and 81% respectively.

CONCLUSION

In the laboratory bioassay, *P. lilacinum* A and *P. lilacinum* B were proven effective in parasitizing *M. incognita* females, eggs and J2 population as well as inhibiting egg hatch of *M. incognita*. Microscopic observation revealed that one of the antagonistic mechanisms of *P. lilacinum* is by proliferation of hyphae on the egg, which then penetrate the egg by mechanical and enzymatic means. Nevertheless, future study should be extended to screen and isolate each metabolite compound produced by *P. lilacinum* to further confirm the pathogenic effect of each respective metabolite towards different growth stage of *M. incognita* and other phytophagous nematodes (egg, juvenile or female).

On the other hand, *B. thuringiensis* crystal and endospores were found ineffective in killing the J2 of *M. incognita* in the laboratory bioassay. It was suspected that the feeding structure (stylet) of *M. incognita* is too small to ingest *B. thuringiensis* toxin or the specific crystal protein genes (Cry6 and Cry14) in *B. thuringiensis* might not be actively expressed or was expressed below the threshold level.

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