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Full Length Article

Isolation of Indigenous Strains of *Paecilomyces lilacinus* with Antagonistic Activity against *Meloidogyne incognita*

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ABSTRACT

Ten indigenous isolates of *Paecilomyces lilacinus* (PL), were isolated from two black pepper farms in Sarawak heavily infested with root-knot nematodes (RKN) as an initiative to control RKN problem. All isolates showed varying degree in colonizing female nematodes. In the female nematode bioassay on water agar, both indigenous strains of PL namely PLA, PLB, and a commercial strain, PLM (as positive control) demonstrated highly significant colonization (>90%, $P \leq 0.01$) on female. In egg parasitism test, spore suspension (10^5 spore/mL) of the strains PLA, PLB and PLM exhibited 78.8%, 66.0% and 73.4% parasitism on eggs, respectively. Meanwhile, hatching of nematode eggs incubated in spore suspension of PLA, PLB and PLM for seven days were significantly reduced; 88-89% of eggs were hatch-inhibited as compared to control (26%). This illustrated both local isolates, PLA and PLB are comparable with PLM as biological control agents for managing RKN infestation on black pepper vines. © 2012 Friends Science Publishers

Key Words: Isolation; *Meloidogyne incognita; Paecilomyces lilacinus;* Biocontrol; Antagonistic; Root-knot nematodes

INTRODUCTION

Black pepper (*Piper nigrum* L.) is an important cash crops of Sarawak, with 6,125 tonnes of black pepper exported from January to June 2010, valued at RM77.889 million (Anonymous, 2010). However, most of black pepper farms in Sarawak are infested by *Meloidogyne spp.* (Kueh, 1978; Leong, 1986; Ravindran, 2000; Eng, 2001), which has become a major constraint to the black pepper production. Survey conducted by Eng (2001) in 43 black pepper farms revealed that root-knot nematodes (RKN) were present in all the farms, implying that black pepper is one of the important hosts of RKN and the problem of RKN infestation in these farms. Currently, no resistant cultivar to RKN is available (Eng, 2001). For the past decades, more attention have been directed to the application of microbial control agents since chemical nematicides are being reappraised in respect of health and environmental concern and limited availability in developing nations (Mukhtar & Pervaz, 2003; Dong & Zhang, 2006).

Paecilomyces lilacinus (PL), a saprophytic soil fungus has drawn many research attentions due to its promising effect in parasitizing and controlling population of phytonematodes (Jatala, 1986; Dube & Smart, 1987; Hewlett *et al.*, 1988; Freitas *et al*., 1995; Nagesh *et al.*, 1997; Khan *et al.*, 2006a; Kiewnick & Sikora, 2006; Brand *et al.*, 2010). It has a high frequency of occurrence in the tropics and subtropic (Morgan *et al*., 1984; Chen *et al*., 1996) and can be found in most of agricultural soils (Brand *et al*., 2010). It has been recognized as a common egg pathogenic fungus of root-knot and cyst nematodes (Rumbos & Kiewnick, 2006). In the study of Eng (2001), 82.9% of the 41 surveyed farms in Sarawak were reported to contain PL despite intensive application of fungicide in the black pepper holdings. Besides, PL has high adaptability in its life strategy, making it competitive in a broad spectrum of range adaptability. It can tolerate wide range of soil pH and able to grow well at 15 -30°C. Cabanillas *et al.* (1989) observed maximum growth of PL at temperature from 24-30ºC and reported its' ability to grow and compete for a wide range of common substrate in soil. The present study aimed to isolate local virulent PL strains and to evaluate their *in vitro* antagonistic activity against different stage of RKN. This may provide opportunity to further evaluate their efficacy in pot and field trial.

MATERIALS AND METHODS

Establishment of pure nematode culture: A pure

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culture of *M. incognita* was established by a single egg mass and maintained on tomato plants (*Lycopersicon esculentum*) raised in pot filled with sterilized pot mixture (2: 1: 1 sand, loam, cocoa peat respectively).

Isolation, screening and identification of PL: Seven strains of PL, designed as PLA, PLEJ1, PLEJ2, PLEK1, PLEK2, PLEK3 and PLEK4 were isolated from egg masses and female nematodes while three strains, designed as PL1A, PLB and PLSA were obtained from soil. PLM, a commercial strain (SUBOTANI^{TM}) 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 (UPM Campus Bintulu, N03°13'00'' E 113°05'56'' & Tondong, N01°26'59''E110°08'2''), 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 *et al.* (2006) before transferred to PDA+ [Potato Dextrose Agar (Merck, Darmstadt, Germany) amended with 0.01% (w/w) Chloramphenicol (Sigma, China) and 3%(w/w) Sodium Chloride] plate. For isolation from soil: Serial dilution and pour plate technique was used (Johnson & Curl, 1972). 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. Identification of PL was based on cultural and morphological characters (Samson, 1975). Axenic cultures were obtained by single spore isolations using fine needle (Johnston & Booth, 1983) and maintained on PDA slants. PLA, PLB and PLM that exhibited highest colonization on female in the later test were further species 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 mass, eggs, egg hatch and Juvenile (J2).

PL mass production: Sterilized substrate (composition: 150 g rice husk, 60 mL distilled water, 10% (w/w) molasses, pH 6.5) packed in polyethylene bag (45 mm x 35 mm) was inoculated with 1 x 10^8 PL conidia. The top of the bag was stuffed up into a cotton-filled piece of PVC pipe (3-cm-diameter & 5-cm-length) that was vertical to the bag. The bag was incubated in the dark at room temperature with periodical shaking (5 min every morning) for aeration and even growth. After 14 days, 5 g of fermented substrate was collected in a sterile test tube, added with 10 mL of 0.05% Tween 80, vortexed for 2 min to dislodge and suspend the spore before being filtered through a doubled layer sterile cheese cloth. Spore concentration was determined using a haemocytometer and was later standardized to 10^5 spores/mL.

Female nematodes bioassay: PLA, PLEJ1, PLEJ2, PLEK1, PLEK2, PLEK3, PLEK4, PL1A, PLB, PLSA and PLM were preliminary screened for their efficacies in colonizing female nematodes. Five equal size females (from pure culture) were extracted from tomato roots, surface sterilized as described before placed at the edge of 5-d-old fungal colony grown on water agar 2% (w/w). Plates were run in triplicate, arranged in a completely randomized design (CRD) and incubated at room temperature. After 4 days, females were observed under stereomicroscope (45x) to detect emerging mycelial from the body surface as sign of colonization.

Colonization r*ate* on female and egg mass: Colonization rate on female by isolate PLA, PLB and PLM was conducted with ten female per plate and four replicate plates per fungus treatment. Sign of colonization was observed daily until the 6th day. Similarly, the procedure was repeated for egg mass.

PL parasitism on eggs: Egg suspension was prepared as described by Nitao *et al.* (1999). A 50 µL of a freshly prepared egg suspension (approximately 300 fresh eggs) was pipetted into a sterile McCaney bottles and added with 1 mL of fungus spore suspension (10^5 spore/mL). Sterile water was added for control treatment. The entire procedure was conducted in aseptic condition. Bottles were sealed with parafilm, arranged in CRD and placed in the dark at room temperature with four replicates 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 (400x) for sign of parasitism.

Effect of spore suspension on egg hatch and Juvenile (J2): Procedure described in egg parasitism test was repeated with fresh material but incubation period was extended to seven days. Egg hatch rate and J2 mortality were determined by counting all eggs, J2 and dead J2 in a counting disc under a stereo-microscope and calculated according to the following formula:

$$\text{Egg hatch rate} = 100 \times J2/(\text{eggs} + J2)$$

$$\text{Juveniles mortality} = 100 \times \text{dead J2/total J2}.$$

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

Statistical analysis: Means of data in percentage were subjected to arc-sine transformation, analyzed 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 ($P<0.01$ & $P<0.05$). Untransformed arithmetic means are reported.

RESULTS AND DISCUSSION

Isolation of PL: Growth of colony on PDA plate formed a basal felt with floccose aerial mycelium giving rise to conidiophores. Diameter of colonies ranged 5-7 cm within 14 days incubation at 28°C ± 2. Conidial heads appeared white but gradually became light brownish when sporulated (Fig. 1). This colour change is consistent with

Table I: Effect of PL spore suspension (10^5 spore/mL) on egg parasitism, egg hatch inhibition and J2 mortality

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

Each value (%) represents the mean of four replicates ± standard error. Values within a raw followed by the same letter are not significantly different according to Duncan Multiple Range Test at P=0.05

Fig. 1: Growth morphology of PL colonies on PDA agar plate

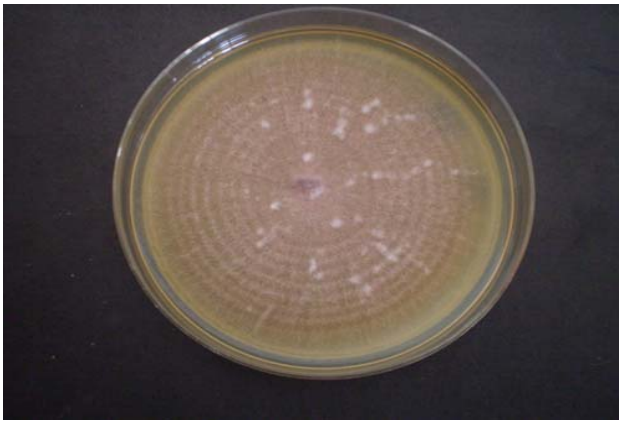
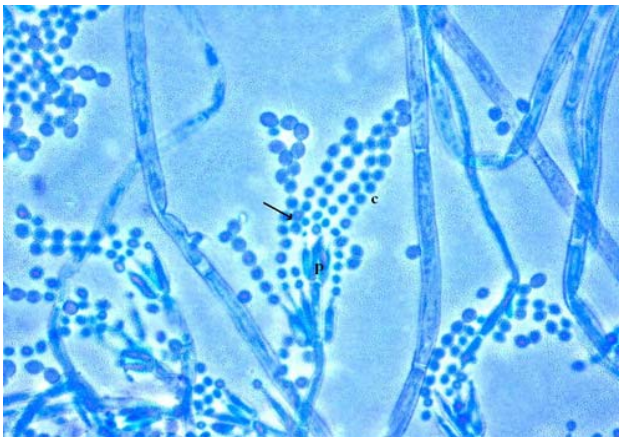


Fig. 2: Hyphae bear phialides (P) with huge numbers of conidia (C) attached loosely in long divergent chains



species of PL reported by Samson (1975). The incorporation of 3% NaCl to PDA gave a suppressive effect on other fungal species, while maintaining the growth and sporulation of PL. Chloramphenicol, a heat stable antibiotic was added to kill a wide range of Gram-negative and Gram-positive bacteria.

Microscopic observation (1000x) revealed conidiophores arise from aerial hyphae, have rough wall, bore verticillate short branches with whorls of 2-4 phialides. The hyphae was hyaline and bear flask shaped phialides (P) with huge numbers of conidia (C) attached

Fig. 3: Colonization on female nematode by commersial strain PLM. Hundreds of conidiosphores (C) radiating from the body surface

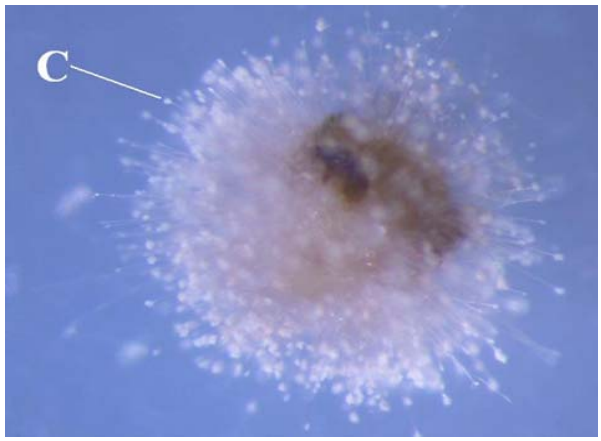
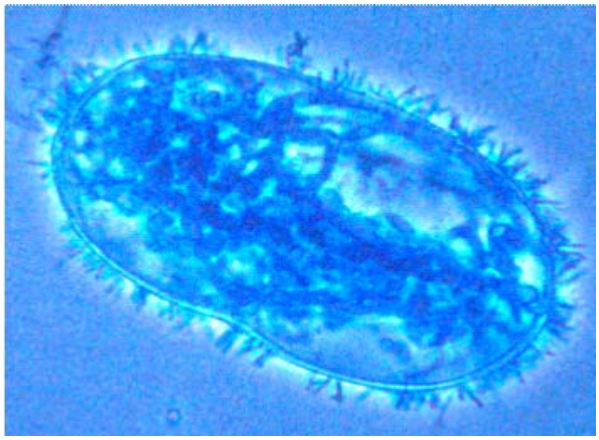


Fig. 4: Hyphae emerged from a deformed shape egg with disintegrated embryo after 4 days of incubation (400 x). It also penetrated into the egg and consumed the egg content



loosely in long divergent chains (Fig. 2). The phialide possessed a swollen basal and a distinct neck. Conidia were in the shape of ellipsoidal to fusiform (Samson, 1975).

Female nematodes bioassay: All PL isolates (Fig. 5) showed varying degrees in colonizing female with PLA, PLB, PLEJ1 and PLM demonstrated the highest significant ($P<0.01$) colonization (>90%), which is consistent with the result of Eapen *et al.* (2005) on the significant parasitism of their PL strain on female nematode. Observation under a stereomicroscope (40x) demonstrated radiating mycelia from the female's body (Fig. 3) unrestricted to vulva, anus or broken opening, which is in concert with the reports of Morgan-Jone *et al.* (1984), Holland *et al.* (1999) and Khan *et al.* (2006b). They suggested that appressoria are not involved in the penetrating process owing to the lack of a tough cross-linked chitin layer on the female body. However, Jatala

Fig. 5: Percentage of female nematodes colonized by PL

Value in each bar represents the mean (%) of three replicates. Bars marked by the same letter are not significantly different according to Duncan Multiple Range Test at P≤0.01

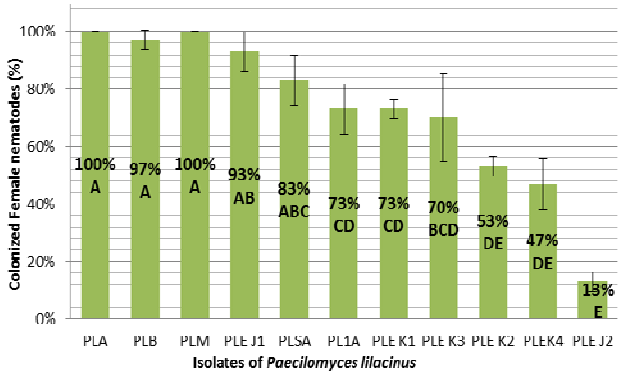
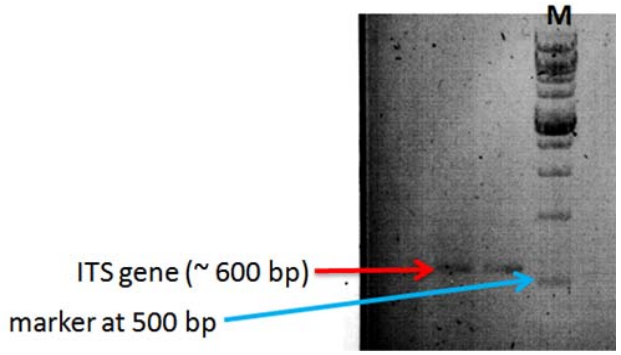


Fig. 5a: Detection of PCR amplification of ITS gene

M represents VC 1kb DNA ladder (Vivantis). For primers ITS1 and ITS4, the size of amplified PCR product was approximately 450-800 bp



(1986) reported a contradictory observation that Peruvian isolate of PL infected female of *M. javanica* only by body opening. Khan *et al.* (2006b) explained that the difference could be due to different profile enzymes of each strain that help in the direct cuticle penetration.

Molecular Identification of PL: For primers EF3 and EF4, the size of amplified PCR product was approximately 1.4-1.5 kb whereas for primers ITS1 and ITS4, the size was approximately 450-800 bp (Fig. 5a). Sequence analysis of PLA clone showed 95-99% similarity to PL sequence in the GenBank, EMBL, DDBJ, PDB database while sequence of PLB clone showed 94% similarity. This molecularly confirmed that both PLA and PLB isolates are of the species PL.

Colonization rate on female and egg mass: Isolate PLA, PLB and PLM did not demonstrate high significant colonization rate on female (Fig. 6). On the 1st day, PLA and PLM demonstrated 50% infection rate followed by PLB with rather low rate (10%). However, all isolates had achieved complete colonization on the 6th day.

Colonization on egg masses by PLA, PLB and PLM was confirmed by observing the emerging mycelial from the surface of egg masses under a stereomicroscope 40X

Fig. 6: Percentage of female nematodes colonized by PL under *in vitro* condition

Dots on each graph line represent mean (% of colonized female) of 4 replicates, marked by the same letter for each respective day are not significantly different according to Duncan Multiple Range Test at P≤0.05. Error bar represent standard error

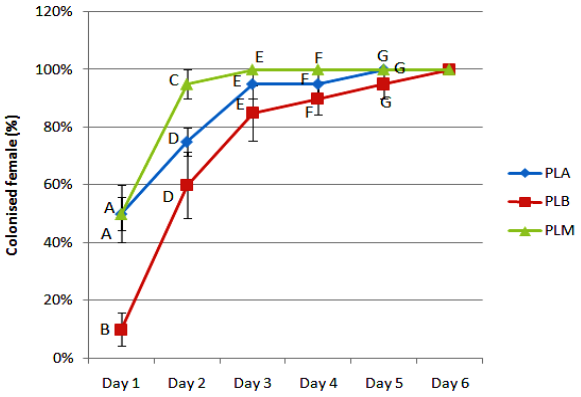
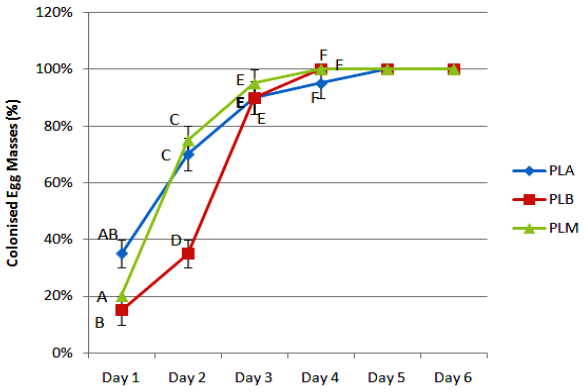


Fig. 7: Percentage of egg masses colonized by PL under *in vitro* condition

Dots on each graph line represent mean (% of colonized egg mass) of 4 replicates, marked by the same letter for each respective day are not significantly different according to Duncan Multiple Range Test at P≤0.05. Error bar represent standard error



(Fig. 7). On the 1st day, PLA, PLB and PLM recorded a colonization rate of 15-35% on egg masses, but drastically increased to 90-95% on the 3rd day and achieved complete colonization on the 5th day. Similarly, all the three isolates showed insignificant colonizing rate on egg mass. The presence of antimicrobial compound (Orion *et al.*, 2001) in gelatinous matrix (GM) of an egg mass protects eggs from microbial infection (Kiewnick *et al.*, 2006). Sharon *et al.* (2007) claimed that an effective parasite should be able to utilize GM as source of nutrient and reproduce in it. Since mycelial was detected on the GM, it is suggested that PLA and PLB possess resistance attribute to antimicrobial compound in the GM, which is in line with those of Zaki and Bhatti (1990) and Eapen *et al.* (2005).

PL parasitism on eggs: PLA, PLM and PLB

demonstrated high and significant parasitism on eggs as compared to control but not significant among themselves, 78.5%, 73.4% and 66.0%, respectively (Table I). This result is in conformity with Sun *et al.* (2006) who reported a high *in vitro* parasitism rate of PL strain YES-X-2-14 on *M. hapla* egg; also Al Kader (2008) who revealed 77% eggs infection after 4 days of incubation. In addition, thirty PL isolates were reported to parasitize 100% of *M. hapla* eggs (Sun *et al.*, 2006).

In this study, early age eggs were more susceptible to PLA and PLB infection than eggs with ready to hatch J2. In most cases, infected eggs contained no J2 with egg content and embryo seemed disintegrated (Fig. 4). This suggested that most immature eggs were parasitized but some eggs containing developing J2 were also infected, and J2 seemed motionless. This is similar to the studies of Irving and Kerry (1986), Jatala (1986), Lopez-Llorca and Duncan (1991) and Eapen *et al.* (2005). However, Morgan-Jone (1984) and Holland *et al.* (1999) claimed that their PL strain infected eggs at all developing stages. This attribute offers advantage in biological control since eggs are the major target of parasitic fungus and can remain dormant in soil for long periods of time (Holland *et al*., 1999).

Under microscopic observation, extensive network of hyphae were detected ramifying several eggs but not growing on other adjacent eggs in the group. At the end of hyphae, a simple swollen hyphae structure, recognized as appressorium appeared pressing onto the eggshell. Some incubated eggs appeared abnormal, deformed and shrunken owing to the pressure exerted by the network of hyphae. This indicates the use of mechanical means to penetrate host, which has been described by Holland *et al.* (1999). When a hyphae encounters an egg surface, it responds thigmotrophically by forming appressoria (Lopez-Llorca *et al.*, 2002), followed by adhesion of an adhesive (an extracellular material on appressoria) to the egg for better binding of fungus to the host (Lopez-Llorca *et al.*, 2008). From these appressoria, the fungus use enzymatic and mechanical means to penetrate the host (Morgan-Jones *et al.*, 1984; Huang *et al.*, 2004; Gortari & Hours, 2008; Lopez-Llorca *et al.*, 2008). According to Morton *et al.* (2004), the secretion of chitinase and proteases by PL facilitated egg penetration by breaking down layers (the barrier) in egg shells so that a narrow infection peg can push through. The combined effect of chitinase and protease produced by PL in degrading eggshell layers of *M. javanica* was proven by Khan *et al.* (2004). The lipid layer disappeared, while the chitin layer was much reduced after enzyme treatment.

Effect of spore suspension on egg hatch inhibition: Egg hatch was not significantly different among the three isolates but significant ($P< 0.05$) with the control 25.9% (Table I). PLM recorded the highest inhibition at (89.4±1.0%), followed by PLA at (88.3±1.4%) and PLB at (88.2±1.7%). Eggs treated with spore suspension appeared shrunken, deformed and with multiple vacuoles. Most eggs in control treatment appeared empty, with many J2 appeared outside the eggs, indicating hatching had occurred. This result is in agreement with Sun *et al.* (2006) who reported average 58% egg hatch inhibition for their 186 PL isolates. Costa *et al.* (2001) reported that culture filtrate of PL grown in Czapek broth greatly reduced egg hatching of MI. According to Bonants *et al.* (1995), hatching of eggs containing mature J2 appeared to be stimulated when incubated in culture filtrate of PL but development of immature eggs appeared to be disrupted.

Effect of spore suspension on J2 mortality: To infect J2, PL needs to overcome the cuticle of nematode. The cuticle is a non-cellular layer production of the hypodermis, which consists of keratin, collagen and fibers (Huang *et al.*, 2004). Once the cuticle is penetrated by fungi hyphae, the nematode get paralyzed, invaded and digested (Tunlid & Jansson, 1991). In this study, spore suspension of PLA, PLB and PLM, demonstrated low mortality effect on J2, 6.0%, 5.5% and 5.7%, respectively with no significant difference ($P< 0.05$). This confirmed the findings of Sun *et al.* (2006) who reported a low average J2 mortality percentage of 16% for their 186 PL isolates. Also, it agrees with the reports of Jatala (1986), Bonants *et al.* (1995), Singh and Mathur (2010) that PL primarily colonized eggs but not juveniles. In contrast, PL strain YES-X-2-14 was reported in exhibiting high *in vitro* nematicidal effect on J2 of *M. hapla* (Sun *et al.*, 2006). Similarly, Al Kader (2008) reported a high nematicidal effect of their PL culture filtrate on J2 of MI, with 99% of J2 immobilized after 2 days of treatment. It is suggested that different strain of PL exhibit different nematicidal effect on J2. Throughout the years, metabolites in culture filtrate of PL have been detected and screened such as paecilotoxin (Mikami *et al*., 1989), acetic acid (Djian *et al.*, 1991) and leucinostatins (Park *et al.*, 2004). These metabolites may potentially cause nematicidal effect toward J2 of nematodes. Culture filtrate of local isolate PLA and PLB grown in PDB recorded acidic pH of 3.30-3.46 (data not shown). It is suspected that both local isolates produced acetic acid, thus proposing a further study to be performed to screen and confirm the active compounds present in the culture filtrate.

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