[biodiv] Submission Acknowledgement

From: Ahmad Dwi Setyawan (smujo.id@gmail.com)

To: igswibawa@yahoo.com

Date: Wednesday, December 11, 2019, 07:38 AM GMT+7

I Gede Swibawa I Gede:

Thank you for submitting the manuscript, "Pur Purpureocillium lilacinum (Thom.) Luangsa Ard. (Syn. Paecilomyces lilacinus (Thom.) Samson.), the Egg Parasites of Guava Root-knot Nematodes in Lampung : -" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/5037
Username: igswibawa

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

Biodiversitas Journal of Biological Diversity

Re: [biodiv] Editor Decision

From: gede swibawa (igswibawa@yahoo.com)

To: sectioneditor1@smujo.id

Date: Monday, January 20, 2020, 09:14 PM GMT+7

Dear Anisa S

Thank your very much for the editor decision information. We will revise as soon as possible.

Regards

IGS

> On Monday, January 20, 2020, 3:50:18 PM GMT+7, Anisa Septiasari <smujo.id@gmail.com> wrote:
>
> I Gede Swibawa I Gede:
>
> We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Purpureocillium lilacinum (Thom.) Luangsa Ard. (Syn. Paecilomyces lilacinus (Thom.) Samson.), the Egg Parasites of Guava Root-knot Nematodes in Lampung ".
>
> Our decision is: Revisions Required
>
> Anisa Septiasari
> sectioneditor1@smujo.id
>
> _____
> Biodiversitas Journal of Biological Diversity

Purpureocillium lilacinum (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.**), the Egg Parasites of Guava Root-knot Nematodes in Lampung**

Abstract

This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have high pathogenic~~ity~~ ability. The exploration of the fungi was carried out in crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and from commercial products in 2016 and 2018. Identification was carried out based on morphological characteristics and molecular~~ly~~ based gene ~~on~~ sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in 5 isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the fungi discovered belong to the group of *Purpureocillium lilacinus* (= *Paecilomyces lillacinus*). In in vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4% - 100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Of all the isolates tested, B01TG had the best ability compared to other isolates. B01TG application was able to infect nematode eggs up to 99.5%, suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity.

Keywords: Guava, *Purpureocillium lilacinum*, root-knot nematode.

Running title: *Purpureocillium lilacinum* nematode egg parasite

Introduction

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. Two species of root-knot nematodes that have been reported to attack guava crystals are *Meloidogyne incognita* and *M. javanica*. According to Swibawa et al. (2017), RKN attack rates range from severe to very severe with a J-2 population> 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and increasing.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala, 1986).

P. lilacinum (syn. *P. lilacinus*) has been used as a biological control agent for nematode in various countries and has shown positive results. Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al., 2006), root-knot nematodes in tomato

Formatted: Font: Italic

plants (Singh et al., 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al., 2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be able to increase the amount and weight of fresh grains of wheat (Hernadez-Leal et al., 2016).

P. lilacinum (Syn. *P. lilacinus*) is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* (syn. *P. lilacinus*) fungi using agar media and Sundaraju & Cannayane (2002) using rice, rice bran, and banana media, while Bran et al. (2009) reproduced this fungus using solid form fermentation. *P. lilacinum* (syn *P. lilacinus*) has been used as an active ingredient of bionematicides and is marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (T. Stanes & Comapny Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum* (syn P. *lilacinus*), such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hooray et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* (syn. *P. lilacinus*) with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to study and molecularly identify the fungal egg parasites of RKN from the exploration of the guava plantation in Lampung.

Materials and Method

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice, namely June-October 2016 and April-September 2018. Exploration in 2016 was carried out in the Crystal guava plantations of PT Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (4.9ºS and 105ºE) and PT GGP PG4 East Lampung (05º00 '- 05º15'S and 105º30' – 105º45'E). Exploration was also carried out from commercial products named (please mention the name of the product and country). Exploration in 2018 was carried out in the Crystal guava plantations of PT GGP PG4 (05°33'55.7964'S) and in Sinar Betung Village, Gunung Alip District, Tanggamus Regency (104º32 '21,516' E) (Figure 1).

Figure 1. Location of the Crystal guava plantation in which the samples of fungal egg parasites of RKN were collected; A = location of PT NTF, East Lampung (05 00 – 05 15 S and 105 30 – 105 45 E); B = location of PT GGP-PG4 Central Lampung (4.9^{0}S and 105^{0}E); C = location of Sinar Betung Village, Tanggamus (05º33’55.7964’’S and 104º32’21.516’’E)

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone (0-20 cm) 20-40 cm away from the base of the stem at four points following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD,

Singapore) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi was separated for isolation purposes. Before isolation, the mass of RKN eggs was dipped in a 1% NaOCl solution for 30 seconds. The fungi were removed using a needle and inoculated on the Potato Sucrose Agar (PSA) medium under a sterile condition in Laminar Air Flow. (How the fungi could be removed from egg mass and inoculated on PSA?)

Identification of fungal egg parasites of root-knot nematodes

Morphological identification. Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica EZ4HD, Singapore) at a magnification of 600-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett & Hunter 1998).

Molecular identification

DNA Extraction. DNA extraction was carried out from fungal cultures aged 2-3 weeks (mention the exact time or age of culture either 2 or 3 weeks). The fungi collected from the Petri dish was suspended with 10 ml of sterile water in a 30 ml centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 ml of 70% chilled ethanol and cooled for 10 minutes at 14,000 rpm. The supernatant was removed, and the sediment in the form of pellet was added with 1 ml extraction buffer (0.5 ml Tris HCl, 1 ml SDS 1% + 2.8 ml NaCl, 0.2 ml Mercaptho Ethanol, 2 ml EDTA, 3.5 ml sterile water). The pellet was moved into a mortar and incubated at -38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) ~~2%~~ was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. 600 µL supernatant was then transferred to a new 1.5 ml tube, and 600 µL Chloroform Isoamyl Alcohol (24: 1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at -40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using centrifuge (Microspin12, Biosan, Latvia).

PCR Amplification. Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/ml). The electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction. The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hal, 1999)Check references. The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al., 2016 missing in reference chapter). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity Test

In vitro test. The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates including BioP from PT GGF Central Lampung, B4120X and B3010 from PT GGP PG4 East Lampung from 2016 exploration, and B412G from PT GGP PG4 East Lampung and B01TG from Tanggamus from 2018 exploration. A drop (± 1 ml) of fungal spore suspension was added to a sterile Petri dish with a diameter of 9 cm at a density of 10^8 spores/ml of potato water solution. Spore suspensions were spread evenly on sterile glass Petri dishes (diameter 9 cm), then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT GGP PG4 East Lampung. An egg mass

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

was taken every 12 hours to be observed under a Leica Brand compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 2). The number of eggs infected with fungus is presented in percent.

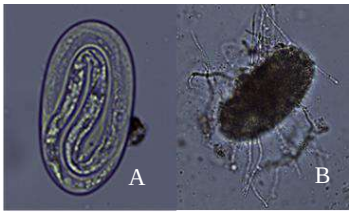


Figure 2. Healthy (A) and infected (B) eggs of ~~*Meloidogyne* spp.~~ Root-knot nematode (RKN)~~:~~ *Meloidogyne* spp.~~A= healthy, B = infected.~~

Greenhouse test. Tomato plants cv. Victoria were used as test plants. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a ~~3 kg~~ polybag filled with 3 kg sterile soil. One day before the tomato plants were planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, namely at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck, 1971). The intensity of root damage was calculated using the formula of Ds = (∑vi x ni) / (N x V) x 100%, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 cc of soil (Hooper et al., 2005 missing in reference chapter). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa, 2004missing in reference chapter). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

Formatted: Font color: Red

Results and Discussion

Results

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 obtained two isolates, all of which came from the Crystal guava plantations of PT GGP PG 4 East Lampung. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. One isolate was obtained from PT GGP PG 1, Terbanggi Besar, Central Lampung. The isolates obtained can be seen in Table 1.

Table 1. Isolates of fungal nematode egg parasites

No	Isolate name	Isolate origin	Location	Year of isolation
1	B4120X	~~Nematode e~~Eggs of *Meloidogyne* spp.	PT GGP PG 1, Central Lapung	2016
2	B3010	Rhizosphere of Crystal guava	PT GGP PG4, East Lampung	
3	BioP	Commercial products (Mention name)	Unknown	
4	B412G	Rhizosphere of Crystal guava	PT GGP PG4, East Lampung	2018
5	B01TG	~~Nematode e~~Eggs of *Meloidogyne* spp.	Tanggamus	

Formatted: Font: Italic

Formatted: Font: Italic

Identity of the isolates of fungal nematode egg parasites

Identification based in morphological characteristics. Based on the morphological characteristics of the colony, the mycelia and spores of the isolates obtained in this study were from the genus of *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony forming air mycelia (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 3A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 3B).

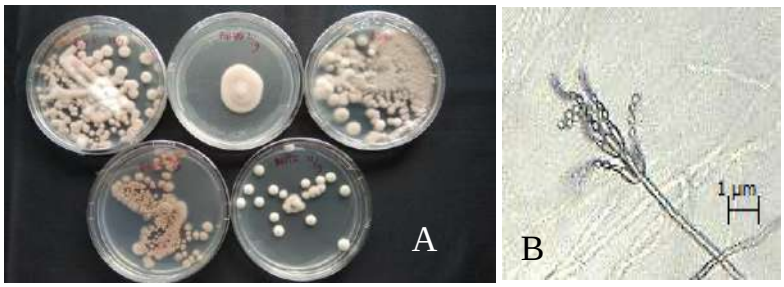


Figure 3. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; A = Colony form; B = the shape of hyphae, conidiophore and conidia (B).

Molecular identification. Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium li~~l~~lacinum*. Based on the phylogenetic tree made, the four isolates found were in the same group with *P. li~~l~~lacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM- L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 4). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

Figure 4. Phylogenetic tree based on the sequencing of ITS 1 – ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). ♦ the isolate of fungal nematode egg parasites discovered.

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. In vitro testing showed that at 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs at a rate of 86.4% to 100%. With a density of 10^8 spores mL-1, the fungi were able to infect as early as 12 ~~hours after infestation (~~HAI~~)~~. B01TG isolates were ~~seen~~ able to infect more than 80% nematode eggs since 12 HAI, while other isolates were ~~seen~~ capable of infecting more than 80% in 36 HAI. 100% infection was achieved in 60 HAI produced by B3010 and B412G isolates (Table 2).

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi~~Hours after infestation (HAI)~~ Hours after infestation (HAI)				
	12	24	36	48	60
	..... %				
Control	0b	0b	0c	0c	0c
BioP	11.3b	11.3b	56.8ab	82.1ab	93.6ab
B4120X	1.1b	1.1b	70.9ab	72.6b	86.4b
B3010	4.9b	12.1b	48.8ab	82.7ab	100.0a
B412G	0.0b	20.9b	34.4bc	89.8a	100.0a
B01TG	81.6a	85.1a	88.6a	93.7a	99.5a
P>F	0.0001**	0.0001**	0.0107**	0.0001**	0.0001**

Remarks: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Formatted Table

The results of the greenhouse test also showed relatively similar results to in vitro testing. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table ~~3~~2). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 cc of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010) - 2571 (control) individuals/5 g roots and 615.2 (B412G) - 1261.6 (control) individuals/300 cc of soil.

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 cc soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Remarks: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The application of *P. lil~~l~~acinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lil~~l~~acinum*, the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4~~3~~).

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66
BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Remarks: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Discussion

Initially, *Purpureocillium lilacinum* was known as *Paecilomyces lilacinus*. In 2011, based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al., (2011) suggested that *Paecilomyces lilacunus* be removed from the genus of *Paecilomyces* and incorporated into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges ~~(Figure 3)~~. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett, 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mention that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explain that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

The identification results based on morphological characteristics were confirmed from the results of molecular identification. BLAST tracing results showed that the five RKN egg parasite isolates obtained in Lampung (B3010, B01TG, BioP, B412G, and B4120X) had high similarity (99%) to the reference strains of *P. lillacinum*. The results of the phylogenetic tree analysis showed that the five isolates belong to the *P. lilacinum* group. These results indicated that the five isolates of RKN egg parasites are *P. lillacinum*.

The *P. lilacinum* fungi isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and the number of infected eggs increased over time ~~(Table 1)~~. Holland et al. (1999) also reported that the number of RKN eggs infected with *P. lilacinum* continued to increase over time. The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported there was a variation in the pathogenicity of *P. lilacinum* (Syn. *P. lilacinus*) fungi isolates against *Meloidogyne* spp. The pathogenicity variation of nematode egg parasite isolates is thought to be influenced by the biophysical conditions of the isolate origin environment. Isolates that have high pathogenicity have the potential to be developed as bionematicides. Fungal egg parasites of nematode are reported to produce protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al., 2004; Bonants et al., 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita* nematodes. In line with what was reported by Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates used had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4 - 100%).

Testing at the greenhouse level also showed that the five *P. lilacinum* fungi isolates had the ability to be root-knot nematode pathogens. The application of fungal suspension in tomato plants infested with root-knot nematodes appeared to be able to reduce J-2 nematode populations in soil and roots ~~(Table 2)~~ and to decrease the root damage of tomato plants ~~(Table 3)~~. Nematode populations in the soil and root damage in plants treated with fungi were lower than J-2 nematode populations in control plants. Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

Acknowledgment

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062 / SP2H / LT / DRPM / 2018 and Contract Pledge No. 393 / UN26.21 / PN / 2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

References

Barnet HL 1969. Ilustrated Genera of Imperfect Fungsi. Second Edition.Burgers Publishing Comapny. USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. Mention title Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal Biological control agents through solid state fermentation: a case study on *Paecilomyces lilacinus* against root-knot nematodes. Micologia Aplicada International. 22(1): 31-48.

Barnett HL, Hunter BB. 1998. *Ilustrated Genera of Imperfect Fungi*. Fourth Edition. Shop APS. USA.

Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Protection 124: 104874 .
https://doi.org/10.1016/j.cropro.2019.104874

Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept. Agric and Consumer Serv. Divsion of palnt industy. No. 23, March-April 1993.

Ecoman Biotch. Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*).. https: // www.en.ecomanbiotech.com / bio-nematicide. diakses Maret 2019

Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.

Holland RJ, Williams KL, Khan A. 1999. Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*. *Nematology*. 1(2): 131-139.

Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. Journal of Entomology and Zoology Studies ~~2018;~~ 6(4): 1700-1704.

Jatala P. 1986. Biological control of plant parasitic nematodes. *Ann. Rev. Phytopathol*. 24: 453-489.

Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode~~s~~, *Pratylenchus thornei*. Tarim Bilimleri Dergisi – Journal of Agricultural Sciences 24: 323-328

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biological Control 31: 346-352.

Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.

Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA & Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50(5):1800-1804.

Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus* . FEMS Microbiol Lett. 321:141–149.

~~Lan X, Zang J, Zong Z, Ma Q, Wang Y. 2017. Evaluation of the biocontrol potential of *Purpureocillium lilacinum* QLP12 againts *Verticillium dahliae* in eggplant. BioMed Research International 2017 : 1-8 https://doi.org/10.1155/2017/4101357~~

~~Lenta BN, Ngatchou J, Frese M, Ladoh-Yameda F, Voundi S, Nardela F, Michalek C, Wibderg D, Ngoulela S, Tsamo E, Kaiser M, Kalinowski J, Seward N. Purpureone, an anthileishmanial ergochrom from the endophytic fungus~~

Formatted: Font color: Auto

Formatted: Font: Italic

Formatted: Font: Italic

Purpureocillium liacinum. De Gruyter Online at 09/28/2016 09:52:04AM : 1-9 Delete this two references as these were not found in the text.

Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolat from mulet gut. Applied and Environmetal Microbiology 57 (7) : 1963-1968.

Paz BAO, Piedrahita OAG, Caycedo JL. 2015. IN VITRO EFFECT OF In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* AND and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root –knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. bol.cient.mus.hist.nat. 19 (2) : 154-172.

Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J. 95 (7-12): 415-417.

Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita.* Biocontrol Science and Technology 23 (12) : 1469-1489.

Stanes T & Comapny Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. Diakses Juni 2017.

Sundararaju P, Cannayane I. 2002. Production of Nematode Egg Parasitic Fungus, *Paecilomyces lilacinus*, on Banana Wastes and Certain Plant Leaves. Indian J. Nematol.32 (2) :183-233.

Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017. Kendari.

Vu D, Groenewald M, de Vries M, Gehrmann T, Stielow B, Eberhardt U, Al-Hatmi A, Groenewald JZ, Cardinali G, Houbraken J, Boekhout T, Crous PW, Robert V and Verkley GJM. 2019. Large-scale generation and analysis of filamentous fungal DNA barcodes boosts coverage for kingdom fungi and reveals thresholdsfor fungal species and higher taxon delimitation. *Stud. Mycol. (92) 135-154. Delete this references*

Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas. Padang.

Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG.24:141–144.

[biodiv] Editor Decision

From: Smujo Editors (smujo.id@gmail.com)

To: igswibawa@yahoo.com; author@smujo.id

Date: Thursday, February 20, 2020, 04:18 PM GMT+7

I GEDE SWIBAWA, YUYUN FITRIANA, SOLIKHIN, RADIX SUHARJO, F.X. SUSILO, EKA RANI, MEI SRI HARYANI, RACHMANSYAH A. WARDANA:

The editing of your submission, "Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/5037

Smujo Editors
editors@smujo.id

Biodiversitas Journal of Biological Diversity

Purpureocillium lilacinum (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.**), the Egg Parasites of Guava Root-knot Nematodes in Lampung**

Abstract. This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have high pathogenic ability. The exploration of the fungi was carried out in crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and from commercial products in 2016 and 2018. Identification was carried out based on morphological characteristics and molecular based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in 5 isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the fungi discovered belong to the group of *Purpureocillium lilacinus* (= *Paecilomyces lillacinus*). In in vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4% - 100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Of all the isolates tested, B01TG had the best ability compared to other isolates. B01TG application was able to infect nematode eggs up to 99.5%, suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity.

Keywords: Guava, *Purpureocillium lilacinum*, root-knot nematode.

Running title: *Purpureocillium lilacinum* nematode egg parasite

INTRODUCTION

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. Two species of root-knot nematodes that have been reported to attack guava crystals are *Meloidogyne incognita* and *M. javanica*. According to Swibawa et al. (2017), RKN attack rates range from severe to very severe with a J-2 population> 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and increasing.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala, 1986).

P. lilacinum (syn. *P. lilacinus*) has been used as a biological control agent for nematode in various countries and has shown positive results. Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al. 2006), root-knot nematodes in tomato plants (Singh et al. 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al.

2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be able to increase the amount and weight of fresh grains of wheat (Hernadez-Leal et al. 2016).

P. lilacinum (Syn. *P. lilacinus*) is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* (syn. *P. lilacinus*) fungi using agar media and Sundaraju & Cannayane (2002) using rice, rice bran, and banana media, while Bran et al. (2009) reproduced this fungus using solid form fermentation. *P. lilacinum* (syn *P. lilacinus*) has been used as an active ingredient of bionematicides and is marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (Stanes & Comapny Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum* (syn P. *lilacinus*), such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hore et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* (syn. *P. lilacinus*) with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to study and molecularly identify the fungal egg parasites of RKN from the exploration of the guava plantation in Lampung.

MATERIALS AND METHODS

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice, namely June-October 2016 and April-September 2018. Exploration in 2016 was carried out in the Crystal guava plantations of PT Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (4.9ºS and 105ºE) and PT GGP PG4 East Lampung (05º00 '- 05º15'S and 105º30' – 105º45'E). Exploration was also carried out from commercial products (Nirama). Exploration in 2018 was carried out in the Crystal guava plantations of PT GGP PG4 (05º33'55.7964'S) and in Sinar Betung Village, Gunung Alip District, Tanggamus Regency (104º32 '21,516' E) (Figure 1).

Figure 1. Location of the Crystal guava plantation in which the samples of fungal egg parasites of RKN were collected; location of PT NTF, East Lampung (05 00 – 05 15 S and 105 30 – 105 45 E) (A); location of PT GGP-PG4 Central Lampung (4.9^0S and 105^0E) (B); location of Sinar Betung Village, Tanggamus (05º33’55.7964’’S and 104º32’21.516’’E) (C)

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone (0-20 cm) 20-40 cm away from the base of the stem at four points following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD,

Singapore) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi was separated for isolation purposes. Before isolation, the root samples containing mass of RKN eggs was dipped in a 1% NaOCl solution for 30 seconds. The root samples were examined under a binocular microscope (Leica EZ4HD, Singapore) at 40-60X magnification to find the mass of RKN eggs which was infected by the fungi (Figure 2). The fungi that infect the egg mass of RKN were taken using a sterile needle and placed in the middle of sterile plastic Petri dish (diameter 9 cm) containing Potato Sucrose Agar (PSA) medium under a sterile condition in Laminar Air Flow.

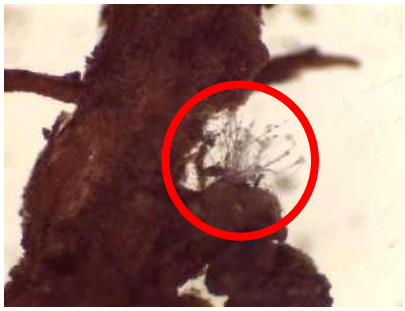


Figure 2. The egg mass of Root Knot Nematode (RKN) which was infected by the fungi.

Identification of fungal egg parasites of root-knot nematodes

Morphological identification. Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica EZ4HD, Singapore) at a magnification of 600-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett & Hunter 1998).

Molecular identification

DNA Extraction. DNA extraction was carried out from fungal cultures aged 3 weeks. The fungi collected from the Petri dish was suspended with 10 mL of sterile water in a 30 mL centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 mL of 70% chilled ethanol and cooled for 10 minutes at 14,000 rpm. The supernatant was removed, and the sediment in the form of pellet was added with 1 mL extraction buffer (0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water). The pellet was moved into a mortar and incubated at -38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. 600 µL supernatant was then transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24: 1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at -40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using centrifuge (Microspin12, Biosan, Latvia).

PCR Amplification. Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction. The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hall 1999). The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al. 2016). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity Test

In vitro test. The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates including BioP from PT GGF Central Lampung, B4120X and B3010 from PT GGP PG4 East Lampung from 2016 exploration, and B412G from PT GGP PG4 East Lampung and B01TG from Tanggamus from 2018 exploration. A drop (± 1 mL) of fungal spore suspension was added to a sterile Petri dish with a diameter of 9 cm at a density of 10^8 spores/mL of potato water solution. Spore suspensions were spread evenly on sterile glass Petri dishes (diameter 9 cm), then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT GGP PG4 East Lampung. An egg mass was taken every 12 hours to be observed under a Leica Brand compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 3). The number of eggs infected with fungus is presented in percent.

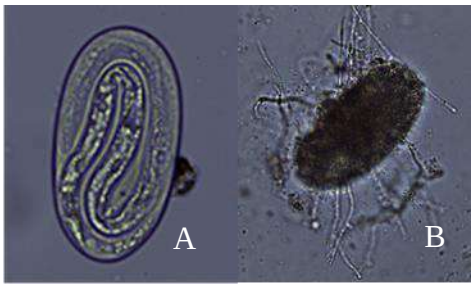


Figure 3. Healthy (A) and infected (B) eggs of Root-knot nematode (RKN) *Meloidogyne* spp.

Greenhouse test. Tomato plants cv. Victoria were used as test plants. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a polybag filled with 3 kg sterile soil. One day before the tomato plants were planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, namely at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck, 1971). The intensity of root damage was calculated using the formula of $Ds = (\sum vi \times ni) / (N \times V) \times 100\%$, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 cc of soil (Hooper et al. 2005). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa 2004). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

RESULTS AND DISCUSSION

Results

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 obtained two isolates, all of which came from the Crystal guava plantations of PT GGP PG 4 East Lampung. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. One isolate was obtained from PT GGP PG 1, Terbanggi Besar, Central Lampung. The isolates obtained can be seen in Table 1.

Table 1. Isolates of fungal nematode egg parasites

No	Isolate name	Isolate origin	Location	Year of isolation
1	B4120X	Eggs of *Meloidogyne* spp.	PT GGP PG 1, Central Lampung	2016
2	B3010	Rhizosphere of Crystal guava	PT GGP PG4, East Lampung	
3	BioP	Commercial products (Nirama)	Unknown	
4	B412G	Rhizosphere of Crystal guava	PT GGP PG4, East Lampung	2018
5	B01TG	Eggs of *Meloidogyne* spp.	Tanggamus	

Identity of the isolates of fungal nematode egg parasites

Identification based in morphological characteristics. Based on the morphological characteristics of the colony, the mycelia and spores of the isolates obtained in this study were from the genus of *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony forming air mycelia (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 4A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 4B).

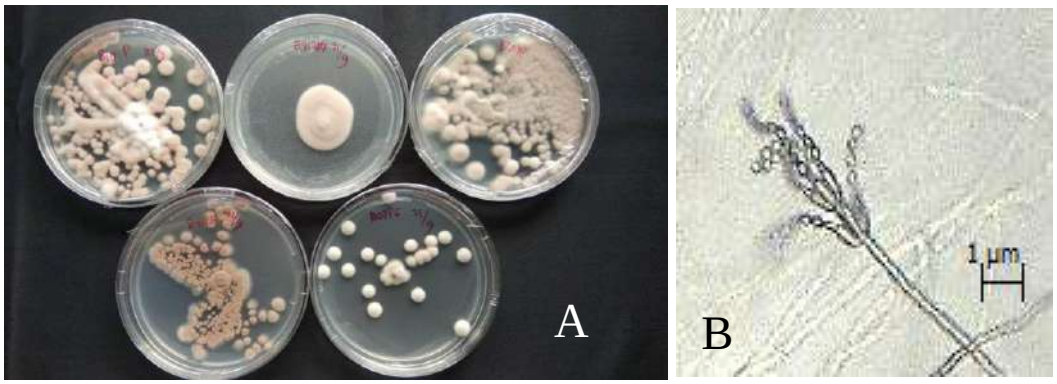


Figure 4. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; Colony form (A); the shape of hyphae, conidiophore and conidia (B).

Molecular identification. Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium lilacinum*. Based on the phylogenetic tree made, the four isolates found were in the same group with *P. lilacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM- L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 4). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

Figure 4. Phylogenetic tree based on the sequencing of ITS 1 – ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). ♦ The isolates of fungal nematode egg parasites discovered.

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. In vitro testing showed that at 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs at a rate of 86.4% to 100%. With a density of 10^8 spores/mL, the fungi were able to infect as early as 12 HAI. B01TG isolates were able to infect more than 80% nematode eggs since 12 HAI, while other isolates were capable of infecting more than 80% in 36 HAI. 100% infection was achieved in 60 HAI produced by B3010 and B412G isolates (Table 2).

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi				
	Hours after infestation (HAI)				
	12	24	36	48	60
	..... %				

Control	0b	0b	0c	0c	0c
BioP	11.3b	11.3b	56.8ab	82.1ab	93.6ab
B4120X	1.1b	1.1b	70.9ab	72.6b	86.4b
B3010	4.9b	12.1b	48.8ab	82.7ab	100.0a
B412G	0.0b	20.9b	34.4bc	89.8a	100.0a
B01TG	81.6a	85.1a	88.6a	93.7a	99.5a
P>F	0.0001**	0.0001**	0.0107**	0.0001**	0.0001**

Remarks: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The results of the greenhouse test also showed relatively similar results to in vitro testing. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table 3). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 cc of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010) - 2571 (control) individuals/5 g roots and 615.2 (B412G) - 1261.6 (control) individuals/300 cc of soil.

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 cc soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Remarks: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The application of *P. lilacinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lilacinum,* the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4).

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66

BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Remarks: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Discussion

Initially, *Purpureocillium lilacinum* was known as *Paecilomyces lilacinus*. In 2011, based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al., (2011) suggested that *Paecilomyces lilacunus* be removed from the genus of *Paecilomyces* and incorporated into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett, 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mention that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explain that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

The identification results based on morphological characteristics were confirmed from the results of molecular identification. BLAST tracing results showed that the five RKN egg parasite isolates obtained in Lampung (B3010, B01TG, BioP, B412G, and B4120X) had high similarity (99%) to the reference strains of *P. lillacinum*. The results of the phylogenetic tree analysis showed that the five isolates belong to the *P. lilacinum* group. These results indicated that the five isolates of RKN egg parasites are *P. lillacinum*.

The *P. lilacinum* fungi isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and the number of infected eggs increased over time. Holland et al. (1999) also reported that the number of RKN eggs infected with *P. lilacinum* continued to increase over time. The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported there was a variation in the pathogenicity of *P. lilacinum* (Syn. *P. lilacinus*) fungi isolates against *Meloidogyne* spp. The pathogenicity variation of nematode egg parasite isolates is thought to be influenced by the biophysical conditions of the isolate origin environment. Isolates that have high pathogenicity have the potential to be developed as bionematicides. Fungal egg parasites of nematode are reported to produce protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al. 2004; Bonants et al. 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita* nematodes. In line with what was reported by Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates used had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4 - 100%).

Testing at the greenhouse level also showed that the five *P. lilacinum* fungi isolates had the ability to be root-knot nematode pathogens. The application of fungal suspension in tomato plants infested with root-knot nematodes appeared to be able to reduce J-2 nematode populations in soil and roots and to decrease the root damage of tomato plants. Nematode populations in the soil and root damage in plants treated with fungi were lower than J-2 nematode populations in control plants. Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062 / SP2H / LT / DRPM / 2018 and Contract Pledge No. 393 / UN26.21 / PN / 2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

REFERENCES

Barnet HL. 1969. Ilustrated Genera of Imperfect Fungsi. Second Edition.Burgers Publishing Comapny. USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal biological control agents through solid state fermentation: a case study on *Paecilomyces lilacinus* against root-knot nematodes. Micol Apl Int. 22(1): 31-48.

Barnett HL, Hunter BB. 1998. *Ilustrated Genera of Imperfect Fungi*. Fourth Edition. Shop APS. USA.

Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Protection 124: 104874.

Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept. Agric and Consumer Serv. Divsion of palnt industry.

Ecoman Biotch. Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*). https: // www.en.ecomanbiotech.com / bio-nematicide. Accessed in March 2019

Gafur A, Swibawa IG. 2004. Methodes in Nematodes and Soil Microbe Research for Belowground Biodiversity Assessment. In: Susilo FX, Gafur A, Utomo M, Evizal R, Murwani S, Swibawa IG. Conservation and Sustainable Management of Below-Ground Biodiversity in Indonesia. Universitas Lampung. Indonesia

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser. 41:95-98.

Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.

Holland RJ, Williams KL, Khan A. 1999. Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*. Nematology. 1(2): 131-139.

Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes. In: Luc M, Sikora RA, Bridge J (Eds.). Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (2nd Edn). CAB International Publisher, UK.

Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. J Entomol Zool Stud. 6(4): 1700-1704.

Jatala P. 1986. Biological control of plant parasitic nematodes. Ann Rev Phytopathol. 24: 453-489.

Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode, *Pratylenchus thornei*. Tarim Bilimleri Dergisi. J Agric Sci. 24: 323-328

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control. 31: 346-352.

Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.

Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA & Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50(5):1800-1804.

Kumar S, Stecher G, Tamura K. 2016. MEGA:7 Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874

Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol Lett. 321:141–149.

Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolat from mulet gut. Appl Environ Microbiol. 57 (7): 1963-1968.

Paz BAO, Piedrahita OAG, Caycedo JL. 2015. In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root –knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. Bol Cient Mus Hist Nat. 19 (2): 154-172.

Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J. 95 (7-12): 415-417.

Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita*. Biocontrol Sci Technol. 23 (12): 1469-1489.

Stanes T & Comapny Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. Accessed in June 2017.

Sundararaju P, Cannayane I. 2002. Production of nematode egg parasitic fungus, *Paecilomyces lilacinus*, on banana wastes and certain plant leaves. Indian J. Nematol.32 (2): 183-233.

Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017. Kendari.

Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas. Padang.

Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG.24:141–144.

Re: [biodiv] Editor Decision

From: gede swibawa (igswibawa@yahoo.com)

To: smujo.id@gmail.com

Date: Friday, February 14, 2020, 04:44 PM GMT+7

Dear Mbak Anisa

We have done a second revised of our manuscript with title of "Purpureocillium lilacinum (Thom.) Luangsa Ard. (Syn. Paecilomyces lilacinus (Thom.) Samson.), the Egg Parasites of Guava Root-knot Nematodes in Lampung ". and we uploaded it on Smujo.

Thank you very much

With Regards

IGS

> On Monday, February 10, 2020, 4:03:50 PM GMT+7, Anisa Septiasari <smujo.id@gmail.com> wrote:
>
> I Gede Swibawa I Gede:
>
> We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Purpureocillium lilacinum (Thom.) Luangsa Ard. (Syn. Paecilomyces lilacinus (Thom.) Samson.), the Egg Parasites of Guava Root-knot Nematodes in Lampung ".
>
> Our decision is: Revisions Required
>
> Anisa Septiasari
> sectioneditor1@smujo.id
>
> _____
> Biodiversitas Journal of Biological Diversity

Morpho-molecular identification and pathogenicity test on fungal parasites ~~*Purpureocillium lilacinum* (Thom.) Luangsa Ard., the egg parasites~~ of guava root-knot nematode eggs in Lampung, Indonesia

Abstract. This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have a high pathogenic ability causing major losses in vegetable crops. The exploration of the fungi was carried out in Cerystal guava plantations in East Lampung, Central Lampung, Tanggamus, and from commercial products in 2016 and 2018. Identification was carried out based on morphological characteristics and molecular based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in a greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in five~~5~~ isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the discovered fungi was~~discovered belong to the group of~~ *Purpureocillium lilacinum* (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.)~~.~~ Based on the~~In~~ in vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4~~%~~ - 100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Among ~~O~~of all the isolates tested, B01TG had the best ability to infect nematode eggs (99.5%), suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity compared to other isolates. ~~B01TG application was able to infect nematode eggs up to 99.5%, suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity~~.

Keywords: Guava, *Purpureocillium lilacinum*, root-knot nematode, ~~tomato plants~~, pathogenicity test.

Running title: Purpureocillium lilacinum nematode egg parasite

INTRODUCTION

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. Two reported species of root-knot nematodes ~~that have been reported to~~ attacking guava crystals are *Meloidogyne incognita* and *M. javanica*. According to Swibawa et al. (2017), all of the RKN attack rates range ~~from~~ is severe ~~to very severe~~ with a second-stage juvenile (J-2) population more than~~>~~ 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and continues to increas~~ing~~.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala, 1986).

Purpureocillium~~*P.*~~ *lilacinum* ~~(syn. *P. lilacinus*)~~ has been used as a biological control agent for nematode in various countries and has shown positive results. Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al. 2006), root-knot nematodes in tomato plants (Singh et al. 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al. 2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be able ~~to~~ increasinge the amount and weight of fresh grains of wheat (Hernadez-Leal et al. 2016).

Commented [A1]: State clearly about this

Commented [A2]: This is species name

Formatted: Font: Italic

Formatted: English (United States)

Commented [A3]: Please revise this

Commented [K4]: Please add the references.

Commented [K5]: Severe = very strict or extreme

Commented [K6]: Please add the references

Purpureocillium~~*P.*~~ *lilacinum* ~~(Syn. *P. lilacinus*)~~ is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* ~~(syn. *P. lilacinus*) fungi~~ using agar media and Sundaraju and~~&~~ Cannayane (2002) using rice, rice bran, and banana media, while Bran et al. (2009) reproduced this fungus using solid form fermentation. *P. lilacinum* ~~(syn *P. lilacinus*)~~ has been used as an active ingredient of bionematicides and is marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (Stanes and~~&~~ Comapany Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum* ~~(syn P. *lilacinus*)~~, such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hore et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* ~~(syn. *P. lilacinus*)~~ with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to study and molecularly identify the fungal egg parasites of RKN from the exploration of the guava plantation in Lampung.

MATERIALS AND METHODS

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice, ~~namely~~ June-October 2016 and April-September 2018. Exploration in 2016 was carried out in the Crystal guava plantations of PT. Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (B) (4.9ºS and 105ºE) and PT. GGP PG4 East Lampung (A) (05º00 '- 05º15'S and 105º30' – 105º45'E). Exploration was also carried out from commercial products (Nirama). Exploration in 2018 was carried out in the Crystal guava plantations of PT. GGP PG4 (05º33'55.7964'S) and in Sinar Betung Village, Gunung Alip District, Tanggamus Regency (C) (104º32 '21,516' E) (Figure 1).

Figure 1. Sampling ~~L~~locations ~~of the Crystal guava plantation in which the samples of fungal egg parasites of RKN were collected; location of PT NTF, East Lampung (05 00 – 05 15 S and 105 30 – 105 45 E) (A); location of PT GGP PG4 Central Lampung (4.9ºS and 105ºE) (B); location of Sinar Betung Village, Tanggamus (05º33'55.7964''S and 104º32'21.516''E) (C)~~

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone (0-20 cm) 20-40 cm away from the base of the stem at four points following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD, ~~Singapore~~Jerman) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi (Fig. 2) was separated for isolation purposes. Before isolation, the root samples containing the mass of RKN eggs wereas dipped in a 1% NaOCl solution for 30 seconds. ~~The root samples were examined under a binocular microscope (Leica EZ4HD, Singapore) at 40-60X magnification to find the mass of RKN eggs which was infected by the fungi (Figure 2).~~ The fungi ~~that~~ infecting the egg mass of RKN were taken using a sterile needle and placed oin the middle

Commented [K7]: Mention it only once when firstly mentioned.

Commented [K8]: Are there any previous studies on the fungal parasites of RKN of guava from Lampung? If yes, there are. Please cited those.

Commented [A9]: What kind of commercial product and where you get them? What is Nirama?
Please state them clearly.

Commented [A10]: Please add the soure of the map

Commented [A11]: ?

Commented [A12]: Leica is product from Jerman.

Commented [A13]: You placedxxxx ccv 777 the fungi on the PSA medium not on petri dish.

of the sterile Potato Sucrose Agar (PSA) medium that put on the sterile plastic pPetri dish (diameter 9 cm) ~~containing Potato Sucrose Agar (PSA) medium~~ under a sterile condition in Laminar Air Flow.

Figure 2. The egg mass of Root Knot Nematode (RKN) which was infected by the fungi.

Commented [K14]: Please zoom in the part of the egg mass of Root Knot Nematode (RKN) which was infected by the fungi

Identification of fungal egg parasites of root-knot nematodes

Morphological identification.

Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica EZ4HD, Singapore) at a magnification of 600-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett and~~&~~ Hunter 1998).

Commented [K15]: Leica EZ4HD is binocular microscope. The zoom range is 8x -35 x.

Commented [A16]: 600 or 400 x?

Molecular identification

DNA Extraction. The DNA extraction was carried out from fungal cultures aged three~~3~~ weeks. The fungi collected from the pPetri dish was suspended with 10 mL of sterile water in a 30 mL centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 mL of 70% chilled ethanol and cooled for 10 minutes at 14,000 rpm. The supernatant was removed, and the ~~sediment in the form of~~ pellet was added with 1 mL extraction buffer (0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water). The pellet was moved into a mortar and incubated at -38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. A total of 600 µL supernatant was then transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24: 1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at -40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using a centrifuge (Microspin12, Biosan, Latvia).

Commented [A17]: Please add the references for this method

Commented [A18]: Is it right that the cooled process for 10 minutesat 14,000 rpm?

PCR Amplification. Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/mL). The

Commented [A19]: Please add the references for this method

electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction. The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hall 1999). The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al. 2016). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity Test

In vitro test. The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates, including BioP from PT. GGF Central Lampung, B4120X and B3010 from PT. GGP PG4 East Lampung from 2016 exploration, and B412G from PT. GGP PG4 East Lampung and B01TG from Tanggamus from 2018 exploration. A drop (± 1 mL) of fungal spore suspension with a density of 10^8 spores/mL of potato water solution was added to a sterile pPetri dish with ~~a~~ diameter of 9 cm. ~~at a density of 10^8 spores/mL of potato water solution~~. Spore suspensions were spread evenly on sterile glass pPetri dishes ~~(diameter 9 cm)~~, then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT. GGP PG4 East Lampung. An egg mass was taken every 12 hours to be observed under a Leica ~~Brand compound~~ compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 3). The number of eggs infected with fungus is presented in percent.

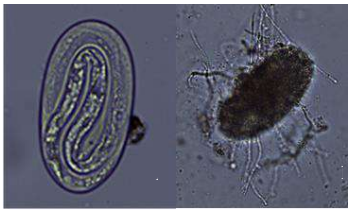


Figure 3. Healthy (A) and infected (B) eggs of Root-knot nematode (RKN) *Meloidogyne* spp.

Greenhouse test. Tomato plants cv. Victoria were used as test plants. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a polybag filled with 3 kg sterile soil. One day before the tomato ~~plants were~~ planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, ~~namely~~ at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck, 1971). The intensity of root damage was calculated using the formula of $Ds = (\sum vi \times ni) / (N \times V) \times 100\%$, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 cc of soil (Hooper et al. 2005). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa 2004). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

Commented [K20]: Please give the abbreviation when first mentioned.

Commented [K21]: Please give A and B to the Figs.

RESULTS AND DISCUSSION

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 in the Crystal guava plantations of PT GGP PG 4 East Lampung obtained two isolates., ~~all of which came from the Crystal guava plantations of PT GGP PG 4 East Lampung~~. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT. GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. Onothere isolate was obtained from PT. GGP PG 1, Terbanggi Besar, Central Lampung. The obtained isolates ~~obtained~~ can be seen in Table 1.

Table 1. The origin of ~~i~~solates of fungal nematode egg parasites

No	Isolate name	Isolate origin	Location	Year of isolation
1	B4120X	Eggs of *Meloidogyne* spp.	PT. GGP PG 1, Central Lampung	2016
2	B3010	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	
3	BioP	Commercial products (Nirama)	Unknown	
4	B412G	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	2018
5	B01TG	Eggs of *Meloidogyne* spp.	Tanggamus	

Identi~~**f**~~**ication**~~**y**~~ **o**~~**f**~~ **the isolates of fungal parasites of nematode egg** ~~**parasite**~~**s**

Identification based in morphological characteristics

Based on the identification, ~~morphological characteristics of the colony,~~ the mycelia and spores of the isolates obtained in this study were species of ~~from~~ the genus ~~of~~ *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony ~~–~~ forming air mycelia (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 4A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 4B).

Figure 4. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; Colony form (A); the shape of hyphae, conidiophore and conidia (B).

Molecular identification

Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium lilacinum*. Based on the phylogenetic tree ~~made~~, the four isolates found were in the same group with *P. lilacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM- L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 4). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

Figure 4. Phylogenetic tree based on the sequencing of ITS 1 – ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). The isolates of fungal nematode egg parasites discovered.

Commented [K22]: Please italicized the Latin name of fungi species

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. In vitro testing showed that at 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs at a rate of 86.4% to 100%. With a density of 10^8 spores/mL, the fungi were able to infect as early as 12 HAI. B01TG isolates were able to infect more than 80% nematode eggs since 12 HAI, while other isolates were capable of infecting more than 80% in 36 HAI. 100% infection was achieved in 60 HAI produced by B3010 and B412G isolates (Table 2).

Commented [K23]: Please rewrite this paragraph. This paragraph is confusing. Otherwise you can delete this part, because you already present the data in the table 2.

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi				
	Hours after infestation (HAI)				
	12	24	36	48	60
	..... %				
Control	0[b]	0[b]	0[c]	0[c]	0[c]
BioP	11.3[b]	11.3[b]	56.8[ab]	82.1[ab]	93.6[ab]
B4120X	1.1[b]	1.1[b]	70.9[ab]	72.6[b]	86.4[b]
B3010	4.9[b]	12.1[b]	48.8[ab]	82.7[ab]	100.0[a]
B412G	0.0[b]	20.9[b]	34.4[bc]	89.8[a]	100.0[a]
B01TG	81.6[a]	85.1[a]	88.6[a]	93.7[a]	99.5[a]
P>F	0.0001[**]	0.0001[**]	0.0107[**]	0.0001[*]	0.0001[**]

Note: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The pathogenicity test ~~results of the greenhouse test~~ in the greenhouse also showed relatively similar results to in vitro test~~ing~~. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table 3). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 cc of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010) - 2571 (control) individuals/5 g roots and 615.2 (B412G) - 1261.6 (control) individuals/300 cc of soil.

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 cc soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The application of *P. lilacinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lilacinum*, the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4).

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66
BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Commented [K24]: Are there any eggs infected by fungi before 12 HAI?

Formatted: Superscript

Discussion

~~Initially,~~ *Purpureocillium lilacinum* was originally described ~~known~~ as *Paecilomyces lilacinus*. ~~BIn 2011, b~~based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al., ~~(~~2011) ~~suggested that~~moved *Paecilomyces lilacunus* ~~be removed from the genus of *Paecilomyces* and incorporated~~ into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal of nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett, 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mentioned that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explained that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

~~The identification results based on morphological characteristics were confirmed from the results of molecular identification. BLAST tracing results showed that the five RKN egg parasite isolates obtained in Lampung (B3010, B01TG, BioP, B412G, and B4120X) had high similarity (99%) to the reference strains of *P. lillacinum*. The results of the phylogenetic tree analysis showed that the five isolates belong to the *P. lilacinum* group. These results indicated that the five isolates of RKN egg parasites are *P. lillacinum*.~~

Purpureocillium ~~The *P.*~~ *lilacinum* ~~fungi~~ isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and after that time, the number of eggs infected by the fungi increase ~~and the number of infected eggs increased over time. Holland et al. (1999) also reported that the number of RKN eggs infected with *P. lilacinum* continued to increase over time.~~ The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported that there was a variation in the pathogenicity of *P. lilacinum* ~~(Syn. *P. lilacinus*)~~ ~~fungi~~ isolates against *Meloidogyne* spp. The pathogenicity variation ~~of nematode egg parasite isolates~~ is thought to be influenced by the biophysical conditions of the ~~isolate origin~~ environmental origin of the isolate. Isolates ~~that have~~with high pathogenicity have the potential to be developed as bio_nematicides. Fungal ~~egg~~ parasites of nematode egg are reported ~~to~~ producinge protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al. 2004; Bonants et al. 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita* ~~nematodes~~. In line with ~~what was reported by~~the research of Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates ~~used~~ had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4 - 100%).

~~T~~Testing at the research in the greenhouse ~~level~~ also showed that the five *P. lilacinum* fungi isolates had the ability to be root-knot nematode pathogens. The application of fungal suspension in tomato plants infested with ~~root-knot nematodes~~RKN ~~appeared to be able to~~can reduce J-2 nematode populations in soil and roots and ~~to~~ decrease the root damage of tomato plants. ~~Nematode populations in the soil and root damage in plants treated with fungi were lower than J-2 nematode populations in control plants.~~ Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

Concluding sentence should be given at the end of the discussion.

Commented [K25]: Already mentioned in the result.

Commented [K26]: Same idea with the previous paragraph.

Commented [A27]: Where?

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062 / SP2H / LT / DRPM / 2018 and Contract Pledge No. 393 / UN26.21 / PN / 2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

REFERENCES

Barnet HL. 1969. Ilustrated Genera of Imperfect Fungsi. Second Edition.Burgers Publishing Comapny. USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal biological control agents through solid state fermentation: Aa case study on *Paecilomyces lilacinus* against root-knot nematodes. Micol Apl Int. 22(1): 31-48.
Barnett HL, Hunter BB. 1998. *Ilustrated Genera of Imperfect Fungi*. Fourth Edition. Shop APS. USA.
Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Protection 124: 104874.
Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept. Agric and Consumer Serv. Division of Pplant Industry.
Ecoman Biotech. Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*). https: // www.en.ecomanbiotech.com / bio-nematicide. Accessed in March 2019
Gafur A, Swibawa IG. 2004. Methodes in Nematodes and Soil Microbe Research for Belowground Biodiversity Assessment. In: Susilo FX, Gafur A, Utomo M, Evizal R, Murwani S, Swibawa IG. Conservation and Sustainable Management of Below-Ground Biodiversity in Indonesia. Universitas Lampung. Indonesia
Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser. 41:95-98.
Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.
Holland RJ, Williams KL, Khan A. 1999. Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*. Nematology. 1(2): 131-139.
Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes. In: Luc M, Sikora RA, Bridge J (Eds.). Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (2nd Edn). CAB International Publisher, UK.
Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. J Entomol Zool Stud. 6(4): 1700-1704.
Jatala P. 1986. Biological control of plant parasitic nematodes. Ann Rev Phytopathol. 24: 453-489.
Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode, *Pratylenchus thornei*. Tarim Bilimleri Dergisi. J Agric Sci. 24: 323-328
Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control. 31: 346-352.
Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.
Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA & Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50(5):1800-1804.
Kumar S, Stecher G, Tamura K. 2016. MEGA:7 Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874
Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol Lett. 321:141–149.
Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolat from mulet gut. Appl Environ Microbiol. 57 (7): 1963-1968.
Paz BAO, Piedrahita OAG, Caycedo JL. 2015. In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al*. and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root –knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. Bol Cient Mus Hist Nat. 19 (2): 154-172.
Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J. 95 (7-12): 415-417.
Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita*. Biocontrol Sci Technol. 23 (12): 1469-1489.
Stanes T & Comapny Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. Accessed in June 2017.
Sundararaju P, Cannayane I. 2002. Production of nematode egg parasitic fungus, *Paecilomyces lilacinus*, on banana wastes and certain plant leaves. Indian J. Nematol. 32 (2): 183-233.
Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017. Kendari. [Indonesian]
Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas. Padang. [Indonesian]
Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG. 24:141–144.

Formatted: English (United States)

Dear Reviewers

Thank you very much for your value reviews and suggestions.

Here are our responses for your comments.

Line 15 : State clearly about this

The sentence has been revised from “The exploration of the fungi was carried out in crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and from commercial products in 2016 and 2018” to “The exploration of the fungi was carried out in 2016 and 2018 from Crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and NirAma, a commercial products which has been used for controlling *Meloydogyne* sp. in Indonesia”

Line 24-25 : This is species name

It has been changed as it is suggested

Line 31 : Please revise this

Has been revised from “Purpureocillium lilacinum nematode egg parasite” to “Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs”

Line 33-34: Please add the references.

Reference has been added

Line 36 : Severe = very strict or extreme

Has been changed to “According to Swibawa et al. (2017), all of the RKN attack rates is severe with a second-stage juvenile (J-2) population more than 1000 individuals per 5 g of roots.”

Line 45: Please add the references

Reference has been added

Line 55: Mention it only once when firstly mentioned

It has been changed as it is suggested

Line 65: Are there any previous studies on the fungal parasites of RKN of guava from Lampung? If yes, there are. Please cited those.

As our understanding, this is the first study on guava-RKN in Lampung.

Line 72: What kind of commercial product and where you get them? What is Nirama? Please state them clearly.

The sentence has been changed to “Exploration was also carried out from NirAma, a commercial products which has been widely used for controlling *Meloidogyne* sp.”

This NirAma is commercial product which has been widely used for controlling some pests including *Meloidogyne* sp.. This commercial product has been used in a research in Indonesia with title “Pengaruh Samping Aplikasi Paecilomyces fumosoroseus Terhadap Semut Hitam, Dolichoderus thoracicus, Predator Helopeltis antonii dan Penggerek Buah Kakao” https://iccri.net/download/Pelita%20Perkebunan/vol_22_no_2_agustus_2006/Pengaruh%20Samping%20Aplikasi%20Paecilomyces%20fumosoroseus%20Terhadap%20Semut%20Hitam,%20Dolichoderus%20thoracicus,%20Predator%20Helopeltis%20antonii%20dan%20Penggerek%20Buah%20Kakao.pdf

This NirAma is also written in commercial product list in the Charnley and Collins. 2007. Entomopathogenic Fungi and Their Role in Pest Control in Esser K. The Mycota. Springer-Verlag Berlin

Table. 10.1. (continued)

Country of the company	Product	Company/*registration other than home country	Target	Active ingredient/ production	Formulation and shelf life	Label crops
	Racer	ditto	Caterpillars on a variety of crops	*B. bassiana*	$1 \times 10^9\ g^{-1}$, 6 months under recommended storage	Rice, cotton, vegetables, chillies, oil seeds, pulses, tea, cardamom
	Biovert Rich	Plantrich (www.plantrich.com)	Sucking insects including aphids, and whitefly	*Verticillium* sp.	$2 \times 10^9\ g^{-1}$	Variety including spices, citrus and horticulture crops
	Pachit Rich	ditto	Thrips, whitefly	*Paecilomyces* sp.	$2 \times 10^9\ ml^{-1}$	Variety including spices, banana, coconut, arecanut
	Bioguard Rich	ditto	Variety of caterpillars	*B. bassiana*	$2 \times 10^9\ ml^{-1}$	
	Biomet Rich	ditto	Variety of insects	*M. anisopliae*	$2 \times 10^9\ ml^{-1}$, liquid formulation	Variety including sugarcane, tea, coffee, spices
Indonesia	Bevaria	Bio Brahma Nusantara, Jakarta, Barat		*B. bassiana*		
	Biometeor	ditto		*M. anisopliae*		
	NirAma	ditto		*P. fumosoroseus*		
Italy	Beavaria brong	Agrifutur s.r.l	Cockchafers	*B. brongniartii*	Barley kernels, 7.5×10^9 conidia g^{-1}, 12 months at 2° C	Variety including grassland, horticulture, fruit
Japan	Biolisa Kamikiri	Nitto Denko	Cerambycid beetles in citrus orchards	*B. brongniartii*	Non-woven fibre bands, impregnated conidia	
Mexico	Bea-Sin	Plant Health Care (www.phcmexico.com.mx)	Various insects	*B. bassiana*		
	Meta-Sin	ditto	Various insects	*M. anisopliae*		
The Netherlands	Mycotal	Koppert B. V. (www.koppert.nl), *Finland, Italy, Norway, Switzerland, Turkey, UK (Denmark Pending)	Whitefly, thrip	*Lecanicillium muscarium*	Wettable powder, $1 \times 10^{10}\ g^{-1}$, 6 months under recommended storage	Various, including glasshouse vegetables and ornamentals
	Vertalec (Fig. 10.2a)	ditto, *Finland, Japan, Norway, Switzerland, UK	Aphids	*L. longisporum*	Blastospores, WP, $1 \times 10^9\ g^{-1}$, 6 months at 3–6 °C	Glasshouse crops
New Zealand	Vertikil	Crop Solutions Ltd. (parent company Millenium Microbes) (www.milleniummicrobes.com)	Thrips, whitefly	Two strains of *L. muscarium*	WP, $1 \times 10^{10}\ g^{-1}$, applied in water with some vegetable oil	Vegetables, flowers under glass. Outdoor crops include kiwifruit and cucurbits

The source has been added

Line 101: ?

The sentence has been changed to ".....root samples were taken using a sucker in the root zone at 20-40 cm of distance from the base of the stem with 0 – 20 cm of soil depth at four cardinal point following the direction of the compass"

Line 104: Leica is product from Jerman.

We have checked the microscope, it was produced in Switzerland.

Line 106-108: You placedxxxx ccv 777 the fungi on the PSA medium not on petri dish.

We have changed the sentence as it is suggested

Line 127: Please zoom in the part of the egg mass of Root Knot Nematode (RKN) which was infected by the fungi

The picture shows the gall containing egg mass RKN which was infected by the fungi. The red circle showed the emerged fungi. Here, the sentence was changed into :

Figure 2. The root gall containing egg mass of Root Knot Nematode (RKN) which was infected by the fungi. The red circle showed the fungi which was emerged

Line 135: Leica EZ4HD is binocular microscope. The zoom range is 8x -35 x.

It has been changed

Line 136: 600 or 400 x?

It has been changed

Line139: Please add the references for this method

We developed this method by ourselves with some optimation.

Line 141: Is it right that the cooled process for 10 minutesat 14,000 rpm?

The sentence has been changed into ".....then added with 1 mL of 70% chilled ethanol and centrifuged for 10 minutes at 14,000 rpm."

Line 157: Please add the references for this method

We developed this method by ourselves with some optimation

Line 175: Please give the abbreviation when first mentioned.

The sentence has been changed into “The treatments were one control and five fungi isolates, including BioP that was isolated from NirAma, a commercial product obtained from PT. Great Giant Plantation (GGP) Plantation Group (PG) 1 Central Lampung in 2016, B4120X and B3010 that were isolated from PT. GGP PG4 East Lampung in 2016, B412G isolated from PT. GGP PG4 East Lampung in 2018 and B01TG that was isolated from Tanggamus in 2018”

Line 197: Please give A and B to the Figs.

It has been added

Line 271: Please italicized the Latin name of fungi species
It has been changed

Line 275: Please rewrite this paragraph. This paragraph is confusing. Otherwise you can delete this part, because you already present the data in the table 2.

It has been changed into “Five isolates of *P. lilacinum* tested proved to have high pathogenicity. At 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs in the range of 86.4% to 100%. The 100% infection was produced by B3010 and B412G isolates (Table 2).”

Line 282: Are there any eggs infected by fungi before 12 HAI?

We could not find any infected egg mass before 12 HAI

Line 329: Already mentioned in the result; Same idea with the previous paragraph.

It has been rvised as it is suggested

Line 331: Same idea with the previous paragraph.

Has been deleted

334-335: Already mentioned in the result.

Has been deleted

Line 345: Concluding sentence should be given at the end of the discussion.

It has been added

Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia

Abstract. This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have a high pathogenic ability causing major losses in vegetable crops . The exploration of the fungi was carried out in 2016 and 2018 from Crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and NirAma, a commercial products which has been used for controlling *Meloydogyne* sp. in Indonesia. Identification was carried out based on morphological characteristics and molecular based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in a greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in five isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the discovered fungi was *Purpureocillium lilacinum* (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.). Based on thein vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4 - 100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Among of all the isolates tested, B01TG had the best ability to infect nematode eggs (99.5%), suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity compared to other isolates. .

Keywords: Guava, *Purpureocillium lilacinum*, root-knot nematode, pathogenicity test.

Running title: Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs

INTRODUCTION

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. (Swibawa et al. 2017). Three reported species of root-knot nematodes attacking guava are *Meloidogyne incognita* (Razak and Lim 1987), *M. Javanica* (Dawabah et al. 2019) and *M. enterolobii* (Kumar and Rawat, 2018). According to Swibawa et al. (2017), all of the RKN attack rates is severe with a second-stage juvenile (J-2) population more than 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and continues to increas.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala, 1986).

Purpureocillium. lilacinum has been used as a biological control agent for nematode in various countries and has shown positive results (Stirling 2014). Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al. 2006), root-knot nematodes in tomato plants (Singh et al. 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al. 2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be ableincreasing the amount and weight of fresh grains of wheat (Hernadez-Leal et al. 2016).

Purpureocillium lilacinum is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* using agar media and Sundaraju and Cannayane (2002) using rice, rice bran, and banana media, while Bran et al. (2009) reproduced this fungus using solid form fermentation. *P. lilacinum* has been used as an active ingredient of bionematicides and is

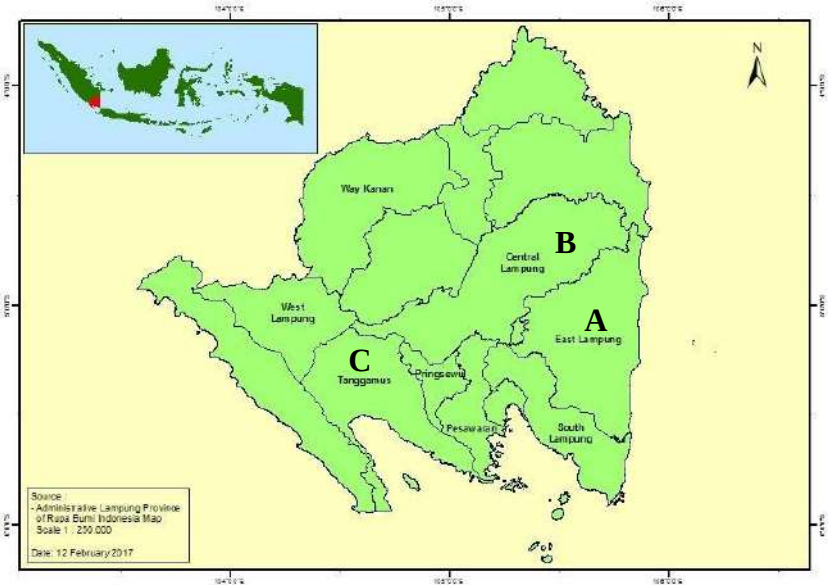
marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (Stanes and Comapany Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum*, such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hore et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to identify and reveal the pathogenicity of fungal egg parasites of RKN from the exploration at the guava plantation in Lampung.

MATERIALS AND METHODS

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice,June-October 2016 and April-September 2018. Exploration in 2016 was carried out in the Crystal guava plantations of PT. Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (B) (4.9ºS and 105ºE) and PT. GGP PG4 East Lampung (A) (05º00 '- 05º15'S and 105º30' – 105º45'E). Exploration was also carried out from NirAma, a commercial products which has been widely used for controlling *Meloidogyne* sp. in Indonesia. Exploration in 2018 was carried out in the Crystal guava plantations of PT. GGP PG4 (05º33'55.7964'S) and in Sinar Betung Village, Gunung Alip District, Tanggamus Regency (C) (104º32 '21,516' E) (Figure 1).



Source: Wulandari et al. (2019)

Figure 1. Sampling locations

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone at 20-40 cm of distance from the base of the stem with 0 – 20 cm of soil depth at four cardinal point following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD, Switzerland) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi (Figure 2) was separated for isolation purposes. Before isolation, the root samples containing the mass of RKN eggs were dipped in a 1% NaOCl solution for 30 seconds. The fungi infecting the egg mass of RKN were taken using a sterile needle and placed on the middle of the sterile Potato Sucrose Agar (PSA) medium that put on the sterile plastic petri dish (diameter 9 cm) under a sterile condition in Laminar Air Flow.

Figure 2. The root gall containing egg mass of Root Knot Nematode (RKN) which was infected by the fungi. The red circle showed the fungi which was emerged

Identification of fungal egg parasites of root-knot nematodes

Morphological identification.

Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica DM500, Switzerland) at a magnification of 400-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett and Hunter 1998).

Molecular identification

DNA Extraction. The DNA extraction was carried out from fungal cultures aged three weeks. The fungi collected from the petri dish was suspended with 10 mL of sterile water in a 30 mL centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 mL of 70% chilled ethanol and centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed, and the pellet was added with 1 mL extraction buffer (0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water). The pellet was moved into a mortar and incubated at -38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. A total of 600 µL supernatant was then transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at -40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using a centrifuge (Microspin12, Biosan, Latvia).

PCR Amplification. Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction. The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hall

1999). The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al. 2016). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity Test

In vitro test. The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates, including BioP that was isolated from NirAma, a commercial product obtained from PT. Great Giant Plantation (GGP) Plantation Group (PG) 1 Central Lampung in 2016, B4120X and B3010 that were isolated from PT. GGP PG4 East Lampung in 2016, B412G isolated from PT. GGP PG4 East Lampung in 2018 and B01TG that was isolated from Tanggamus in 2018. A drop (± 1 mL) of fungal spore suspension with a density of 10^8 spores/mL of potato water solution was added to a sterile petri dish with diameter of 9 cm. . Spore suspensions were spread evenly on sterile glass petri dishes, then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT. GGP PG4 East Lampung. An egg mass was taken every 12 hours to be observed under a Leica compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 3). The number of eggs infected with fungus is presented in percent.

Figure 3. Healthy (A) and infected (B) eggs of Root-knot nematode (RKN) *Meloidogyne* sp.

Greenhouse test. Tomato plants cv. Victoria were used as test plants. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a polybag filled with 3 kg sterile soil. One day before the tomato planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck, 1971). The intensity of root damage was calculated using the formula of $Ds = (\sum vi \times ni) / (N \times V) \times 100\%$, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 cc of soil (Hooper et al. 2005). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa 2004). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

RESULTS AND DISCUSSION

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 in the Crystal guava plantations of PT GGP PG 4 East Lampung obtained two isolates. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT. GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. Onother isolate was obtained from PT. GGP PG 1, Terbanggi Besar, Central Lampung. The obtained isolates can be seen in Table 1.

Table 1. The origin of isolates of fungal nematode egg parasites

No	Isolate name	Isolate origin	Location	Year of isolation
1	B4120X	Eggs of *Meloidogyne* spp.	PT. GGP PG 1, Central Lampung	2016
2	B3010	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	
3	BioP	Commercial products (NirAma)	Unknown	
4	B412G	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	2018
5	B01TG	Eggs of *Meloidogyne* spp.	Tanggamus	

Identification on the isolates of fungal parasites of nematode eggs

Identification based in morphological characteristics

Based on the identification, the mycelia and spores of the isolates obtained in this study were species of the genus *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony-forming air mycelia (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 4A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 4B).

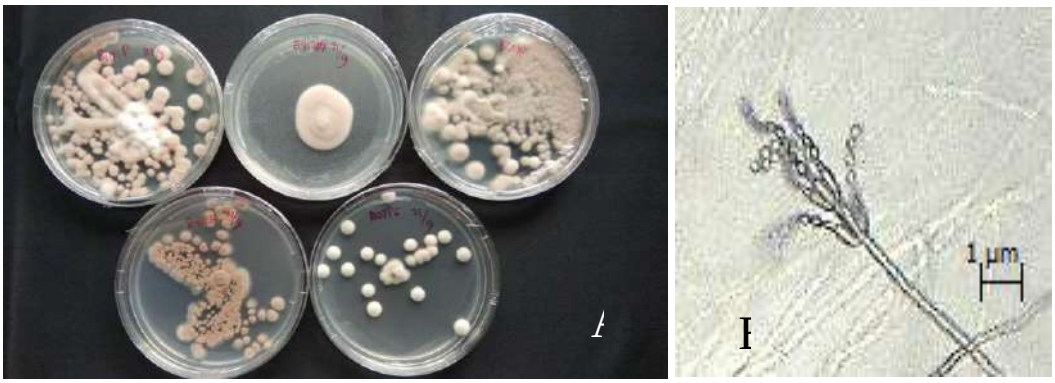


Figure 4. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; Colony form (A); the shape of hyphae, conidiophore and conidia (B).

Molecular identification

Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium lilacinum*. Based on the phylogenetic tree, the four isolates found were in the same group with *P. lilacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM- L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 4). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

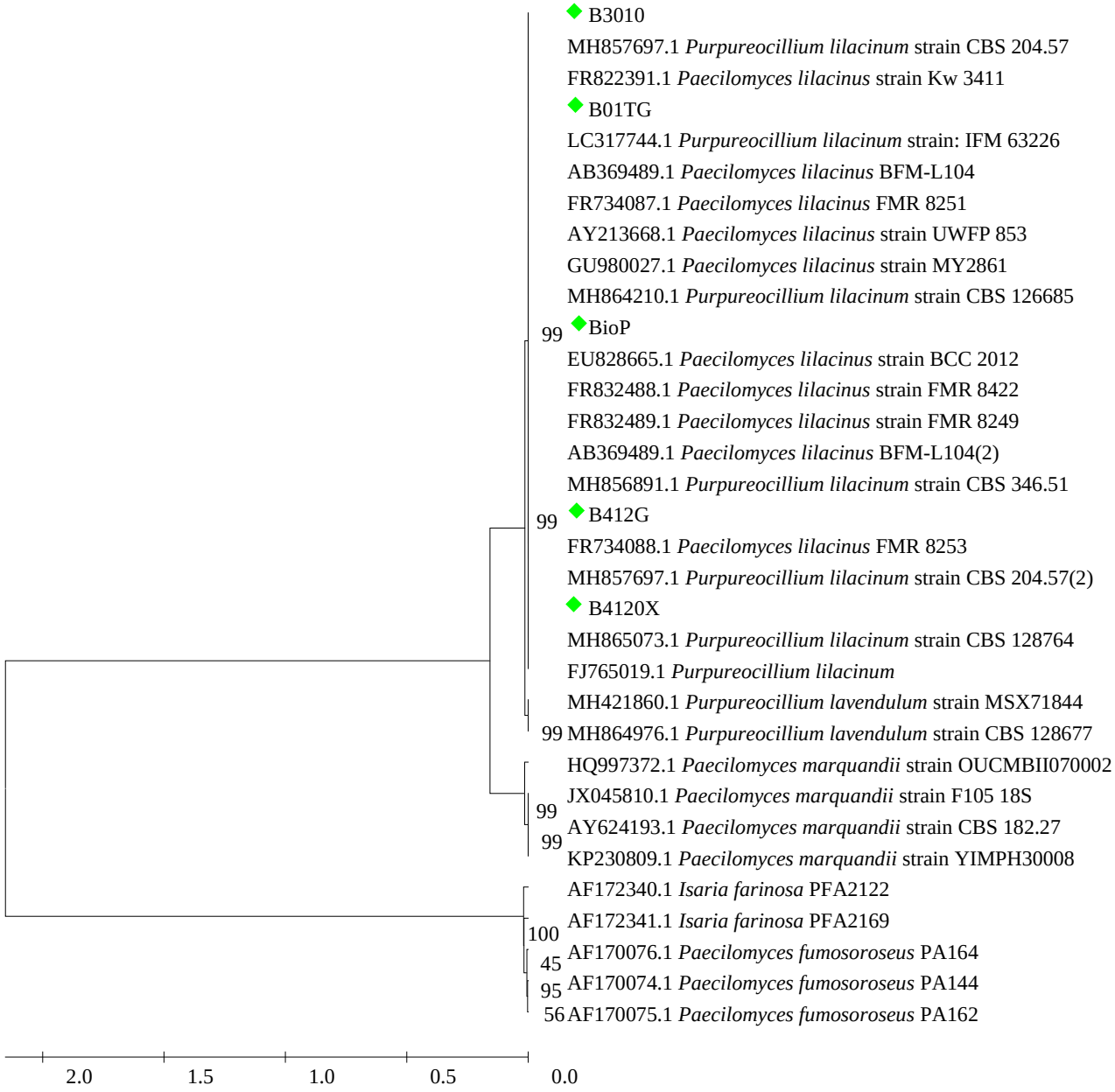


Figure 4. Phylogenetic tree based on the sequencing of ITS 1 – ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). ◆The isolates of fungal nematode egg parasites discovered.

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. At 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs in the range of 86.4% to 100%. The 100% infection was produced by B3010 and B412G isolates (Table 2).

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi				
	Hours after infestation (HAI)				
	12	24	36	48	60
	……. % ……				
Control	0^{b}	0^{b}	0^{c}	0^{c}	0^{c}
BioP	11.3^{b}	11.3^{b}	56.8^{ab}	82.1^{ab}	93.6^{ab}
B4120X	1.1^{b}	1.1^{b}	70.9^{ab}	72.6^{b}	86.4^{b}
B3010	4.9^{b}	12.1^{b}	48.8^{ab}	82.7^{ab}	100.0^{a}
B412G	0.0^{b}	20.9^{b}	34.4^{bc}	89.8^{a}	100.0^{a}
B01TG	81.6^{a}	85.1^{a}	88.6^{a}	93.7^{a}	99.5^{a}
P>F	0.0001^{**}	0.0001^{**}	0.0107^{**}	0.0001^{*}*	0.0001^{**}

Note: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The pathogenicity test in the greenhouse also showed relatively similar results to in vitro test. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table 3). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 cc of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010) - 2571 (control) individuals/5 g roots and 615.2 (B412G) - 1261.6 (control) individuals/300 cc of soil.

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 cc soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The application of *P. lilacinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lilacinum*, the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4).

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66
BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Discussion

Purpureocillium lilacinum was originally described as *Paecilomyces lilacinus*. Based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al. (2011) moved *Paecilomyces lilacunus* into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal of nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett, 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mentioned that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explained that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

Purpureocillium lilacinum isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and after that time, the number of eggs infected by the fungi increased. The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported that there was a variation in the pathogenicity of *P. lilacinum* isolates against *Meloidogyne* spp. The pathogenicity variation is thought to be influenced by the biophysical conditions of the environmental origin of the isolate. Isolates with high pathogenicity have the potential to be developed as bio nematicides. Fungal parasites of nematode egg are reported producing protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al. 2004; Bonants et al. 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita*. In line with the research of Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4 - 100%).

The research in the greenhouse also showed that the five *P. lilacinum* fungi had the ability to be pathogen of root-knot nematode. The application of fungal suspension in tomato plants infested with RKN can reduce J-2 nematode populations in soil and roots and decrease the root damage of tomato plants (unpublished data). Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

Based on the result, it can be concluded that the five fungal isolates used in this study were *P. lillacunum*. All the fungal isolates showed good capability to be used as biological control agent RKN, which were resulted more than 80% of eggs mass infection.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062 / SP2H / LT / DRPM / 2018 and Contract Pledge No. 393 / UN26.21 / PN / 2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

REFERENCES

Barnet HL. 1969. Ilustrated Genera of Imperfect Fungsi. Second Edition.Burgers Publishing Comapny. USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal biological control agents through solid state fermentation: A case study on *Paecilomyces lilacinus* against root-knot nematodes. Micol Apl Int 22(1): 31-48.

Barnett HL, Hunter BB. 1998. *Ilustrated Genera of Imperfect Fungi*. Fourth Edition. Shop APS. USA.

Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Prot 124: 104874.

Dawabah AAM, Al-Yahya FA and Lafi HA. 2019. Integrated management of plant-parasitic nematodes on guava and fig trees under tropical field conditions. Egypt J Biol Pest Co. https://doi.org/10.1186/s41938-019-0133-9

Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept. Agric and Consumer Serv. Division of Plant Industry.

Ecoman Biotech. Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*). https: // www.en.ecomanbiotech.com / bio-nematicide. Accessed in March 2019

Gafur A, Swibawa IG. 2004. Methodes in Nematodes and Soil Microbe Research for Belowground Biodiversity Assessment. In: Susilo FX, Gafur A, Utomo M, Evizal R, Murwani S, Swibawa IG. Conservation and Sustainable Management of Below-Ground Biodiversity in Indonesia. Universitas Lampung. Indonesia

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.

Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.

Holland RJ, Williams KL, Khan A. 1999. Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*.Nematology 1(2): 131-139.

Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes. In: Luc M, Sikora RA, Bridge J (Eds.). Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (2nd Edn). CAB International Publisher, UK.

Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. J Entomol Zool Stud 6(4): 1700-1704.

Jatala P. 1986. Biological control of plant parasitic nematodes. Ann Rev Phytopathol 24: 453-489.

Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode, *Pratylenchus thornei*. Tarim Bilimleri Dergisi. J Agric Sci 24: 323-328

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control 31: 346-352.

Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.

Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA & Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50(5):1800-1804.

Kumar S, Stecher G, Tamura K. 2016. MEGA:7 Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874

Kumar S, Rawat S. 2018. First report on the root-knot nematode *Meloidogyne enterolobii* (Yang and Eisenback, 1988) infecting guava (*Psidium guajava*) in Udham Singh Nagar of Uttarakhand, India. Int J Curr Microbiol App Sci 7(4): 1720-1724.

Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol Lett 321:141–149.

Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolat from mulet gut. Appl Environ Microbiol 57 (7): 1963-1968.

Paz BAO, Piedrahita OAG, Caycedo JL. 2015. In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root –knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. Bol Cient Mus Hist Nat 19 (2): 154-172.

Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J 95 (7-12): 415-417.

Razak AR, Lim TK. 1987. Occurrence of the root-knot nematode Meloidogyne incognita on guava in Malaysi. Pertanika. 10(3): 265-270

Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita.* Biocontrol Sci Technol 23 (12): 1469-1489.

Stanes T & Comapny Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. Accessed in June 2017.

Stirling GR. 2014. Biological Control of Plant-parasitic Nematodes (2nd Edn). CAB International Publisher. UK

Sundararaju P, Cannayane I. 2002. Production of nematode egg parasitic fungus, *Paecilomyces lilacinus,* on banana wastes and certain plant leaves. Indian J. Nematol 32 (2): 183-233.

Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017. Kendari. [Indonesian]

Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas. Padang. [Indonesian]

Wulandari C, Budiono P, Ekayani M. 2019. Impacts of the new decentralization Law 23/2014 to the implementation of community based forest management in Lampung Province, Indonesia. IOP Conf. Series: Earth and Environmental Science. 285 (2019) 012006. https://doi:10.1088/1755-1315/285/1/012006

475 Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG
476 24:141–144.

Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia

Abstract. This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have a high pathogenic ability causing major losses in vegetable crops . The exploration of the fungi was carried out in 2016 and 2018 from Crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and NirAma, a commercial products which has been used for controlling *Meloydogyne* sp. in Indonesia. Identification was carried out based on morphological characteristics and molecular based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in a greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in five isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the discovered fungi was *Purpureocillium lilacinum* (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.). Based on thein vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4 - 100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Among of all the isolates tested, B01TG had the best ability to infect nematode eggs (99.5%), suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity compared to other isolates. .

Keywords: Guava, *Purpureocillium lilacinum*, root-knot nematode, pathogenicity test.

Running title: Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs

INTRODUCTION

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. (Swibawa et al. 2017). Three reported species of root-knot nematodes attacking guava are *Meloidogyne incognita* (Razak and Lim 1987), *M. Javanica* (Dawabah et al. 2019) and *M. enterolobii* (Kumar and Rawat, 2018). According to Swibawa et al. (2017), all of the RKN attack rates is severe with a second-stage juvenile (J-2) population more than 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and continues to increas.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala, 1986).

Purpureocillium. lilacinum has been used as a biological control agent for nematode in various countries and has shown positive results (Stirling 2014). Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al. 2006), root-knot nematodes in tomato plants (Singh et al. 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al. 2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be ableincreasing the amount and weight of fresh grains of wheat (Hernadez-Leal et al. 2016).

Purpureocillium lilacinum is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* using agar media and Sundaraju and Cannayane (2002) using rice, rice bran, and banana media, while Bran et al. (2009) reproduced this fungus using solid form fermentation. *P. lilacinum* has been used as an active ingredient of bionematicides and is

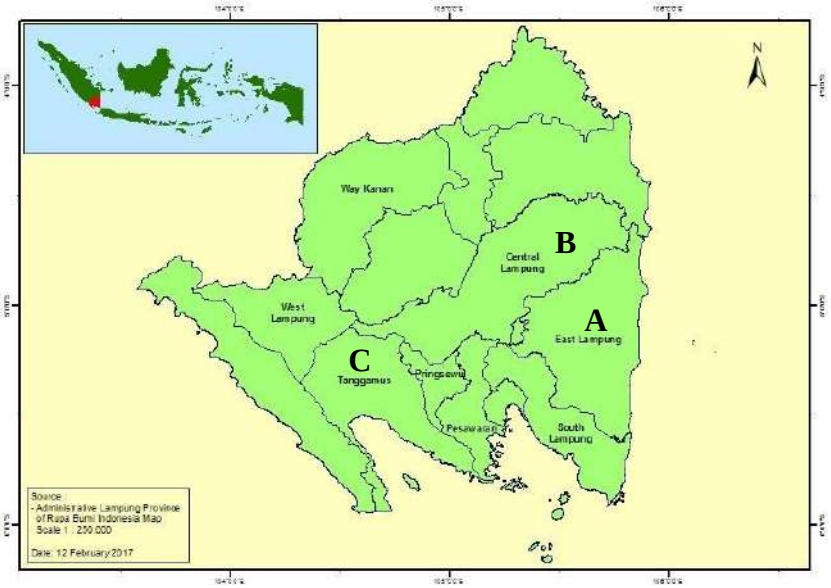
marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (Stanes and Comapany Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum*, such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hore et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to identify and reveal the pathogenicity of fungal egg parasites of RKN from the exploration at the guava plantation in Lampung.

MATERIALS AND METHODS

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice,June-October 2016 and April-September 2018. Exploration in 2016 was carried out in the Crystal guava plantations of PT. Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (B) (4.9ºS and 105ºE) and PT. GGP PG4 East Lampung (A) (05º00 '- 05º15'S and 105º30' – 105º45'E). Exploration was also carried out from NirAma, a commercial products which has been widely used for controlling *Meloidogyne* sp. in Indonesia. Exploration in 2018 was carried out in the Crystal guava plantations of PT. GGP PG4 (05º33'55.7964'S) and in Sinar Betung Village, Gunung Alip District, Tanggamus Regency (C) (104º32 '21,516' E) (Figure 1).



Source: Wulandari et al. (2019)

Figure 1. Sampling locations

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone at 20-40 cm of distance from the base of the stem with 0 – 20 cm of soil depth at four cardinal point following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD, Switzerland) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi (Figure 2) was separated for isolation purposes. Before isolation, the root samples containing the mass of RKN eggs were dipped in a 1% NaOCl solution for 30 seconds. The fungi infecting the egg mass of RKN were taken using a sterile needle and placed on the middle of the sterile Potato Sucrose Agar (PSA) medium that put on the sterile plastic petri dish (diameter 9 cm) under a sterile condition in Laminar Air Flow.

Figure 2. The root gall containing egg mass of Root Knot Nematode (RKN) which was infected by the fungi. The red circle showed the fungi which was emerged

Identification of fungal egg parasites of root-knot nematodes

Morphological identification.

Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica DM500, Switzerland) at a magnification of 400-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett and Hunter 1998).

Molecular identification

DNA Extraction. The DNA extraction was carried out from fungal cultures aged three weeks. The fungi collected from the petri dish was suspended with 10 mL of sterile water in a 30 mL centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 mL of 70% chilled ethanol and centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed, and the pellet was added with 1 mL extraction buffer (0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water). The pellet was moved into a mortar and incubated at -38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. A total of 600 µL supernatant was then transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at -40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using a centrifuge (Microspin12, Biosan, Latvia).

PCR Amplification. Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction. The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hall

1999). The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al. 2016). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity Test

In vitro test. The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates, including BioP that was isolated from NirAma, a commercial product obtained from PT. Great Giant Plantation (GGP) Plantation Group (PG) 1 Central Lampung in 2016, B4120X and B3010 that were isolated from PT. GGP PG4 East Lampung in 2016, B412G isolated from PT. GGP PG4 East Lampung in 2018 and B01TG that was isolated from Tanggamus in 2018. A drop (± 1 mL) of fungal spore suspension with a density of 10^8 spores/mL of potato water solution was added to a sterile petri dish with diameter of 9 cm. . Spore suspensions were spread evenly on sterile glass petri dishes, then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT. GGP PG4 East Lampung. An egg mass was taken every 12 hours to be observed under a Leica compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 3). The number of eggs infected with fungus is presented in percent.

Figure 3. Healthy (A) and infected (B) eggs of Root-knot nematode (RKN) *Meloidogyne* sp.

Greenhouse test. Tomato plants cv. Victoria were used as test plants. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a polybag filled with 3 kg sterile soil. One day before the tomato planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck, 1971). The intensity of root damage was calculated using the formula of $Ds = (\sum vi \times ni) / (N \times V) \times 100\%$, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 cc of soil (Hooper et al. 2005). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa 2004). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

RESULTS AND DISCUSSION

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 in the Crystal guava plantations of PT GGP PG 4 East Lampung obtained two isolates. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT. GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. Onother isolate was obtained from PT. GGP PG 1, Terbanggi Besar, Central Lampung. The obtained isolates can be seen in Table 1.

Table 1. The origin of isolates of fungal nematode egg parasites

No	Isolate name	Isolate origin	Location	Year of isolation
1	B4120X	Eggs of *Meloidogyne* spp.	PT. GGP PG 1, Central Lampung	2016
2	B3010	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	
3	BioP	Commercial products (NirAma)	Unknown	
4	B412G	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	2018
5	B01TG	Eggs of *Meloidogyne* spp.	Tanggamus	

Identification on the isolates of fungal parasites of nematode eggs

Identification based in morphological characteristics

Based on the identification, the mycelia and spores of the isolates obtained in this study were species of the genus *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony-forming air mycelia (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 4A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 4B).

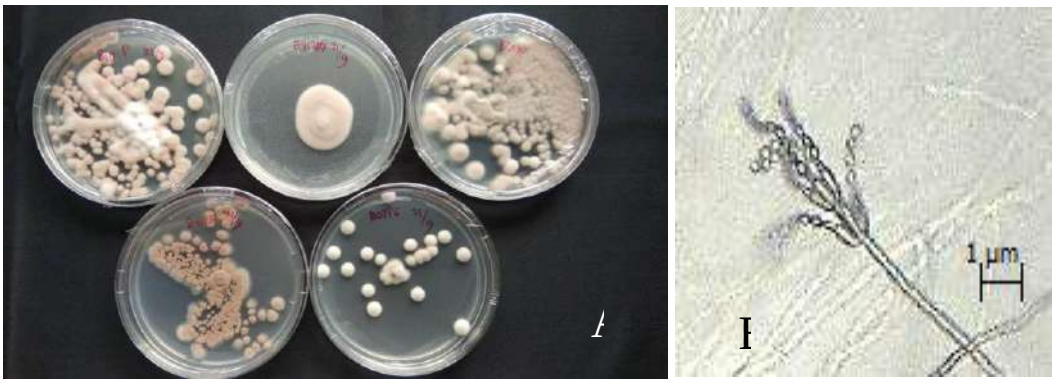


Figure 4. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; Colony form (A); the shape of hyphae, conidiophore and conidia (B).

Molecular identification

Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium lilacinum*. Based on the phylogenetic tree, the four isolates found were in the same group with *P. lilacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM- L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 4). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

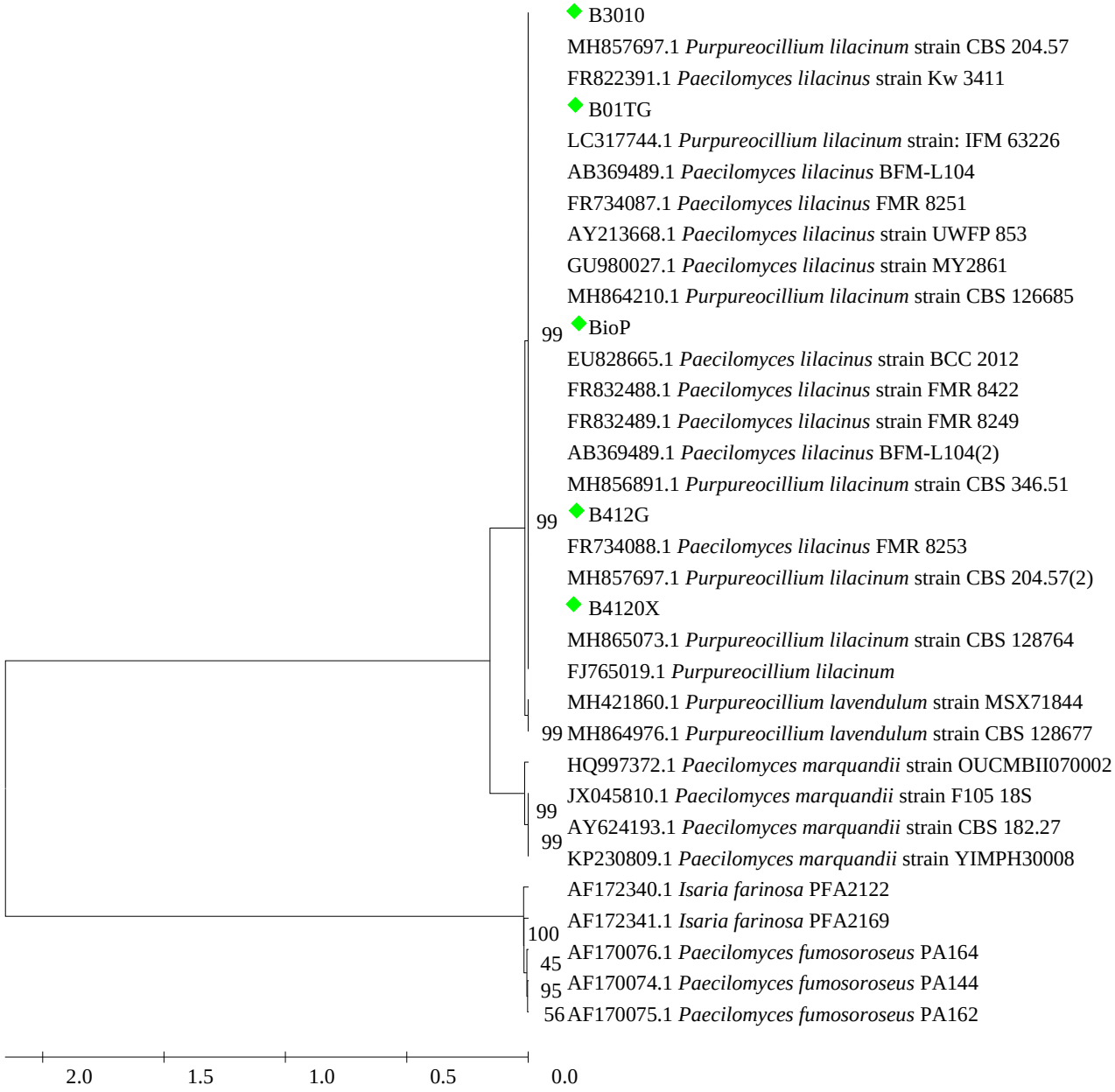


Figure 4. Phylogenetic tree based on the sequencing of ITS 1 – ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). ◆The isolates of fungal nematode egg parasites discovered.

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. At 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs in the range of 86.4% to 100%. The 100% infection was produced by B3010 and B412G isolates (Table 2).

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi				
	Hours after infestation (HAI)				
	12	24	36	48	60
	……. % ……				
Control	0^{b}	0^{b}	0^{c}	0^{c}	0^{c}
BioP	11.3^{b}	11.3^{b}	56.8^{ab}	82.1^{ab}	93.6^{ab}
B4120X	1.1^{b}	1.1^{b}	70.9^{ab}	72.6^{b}	86.4^{b}
B3010	4.9^{b}	12.1^{b}	48.8^{ab}	82.7^{ab}	100.0^{a}
B412G	0.0^{b}	20.9^{b}	34.4^{bc}	89.8^{a}	100.0^{a}
B01TG	81.6^{a}	85.1^{a}	88.6^{a}	93.7^{a}	99.5^{a}
P>F	0.0001^{**}	0.0001^{**}	0.0107^{**}	0.0001^{*}*	0.0001^{**}

Note: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The pathogenicity test in the greenhouse also showed relatively similar results to in vitro test. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table 3). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 cc of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010) - 2571 (control) individuals/5 g roots and 615.2 (B412G) - 1261.6 (control) individuals/300 cc of soil.

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 cc soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

The application of *P. lilacinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lilacinum*, the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4).

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66
BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Discussion

Purpureocillium lilacinum was originally described as *Paecilomyces lilacinus*. Based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al. (2011) moved *Paecilomyces lilacunus* into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal of nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett, 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mentioned that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explained that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

Purpureocillium lilacinum isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and after that time, the number of eggs infected by the fungi increased. The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported that there was a variation in the pathogenicity of *P. lilacinum* isolates against *Meloidogyne* spp. The pathogenicity variation is thought to be influenced by the biophysical conditions of the environmental origin of the isolate. Isolates with high pathogenicity have the potential to be developed as bio nematicides. Fungal parasites of nematode egg are reported producing protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al. 2004; Bonants et al. 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita*. In line with the research of Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4 - 100%).

The research in the greenhouse also showed that the five *P. lilacinum* fungi had the ability to be pathogen of root-knot nematode. The application of fungal suspension in tomato plants infested with RKN can reduce J-2 nematode populations in soil and roots and decrease the root damage of tomato plants (unpublished data). Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

Based on the result, it can be concluded that the five fungal isolates used in this study were *P. lillacunum*. All the fungal isolates showed good capability to be used as biological control agent RKN, which were resulted more than 80% of eggs mass infection.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062 / SP2H / LT / DRPM / 2018 and Contract Pledge No. 393 / UN26.21 / PN / 2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

REFERENCES

Barnet HL. 1969. Ilustrated Genera of Imperfect Fungsi. Second Edition.Burgers Publishing Comapny. USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal biological control agents through solid state fermentation: A case study on *Paecilomyces lilacinus* against root-knot nematodes. Micol Apl Int 22(1): 31-48.

Barnett HL, Hunter BB. 1998. *Ilustrated Genera of Imperfect Fungi*. Fourth Edition. Shop APS. USA.

Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Prot 124: 104874.

Dawabah AAM, Al-Yahya FA and Lafi HA. 2019. Integrated management of plant-parasitic nematodes on guava and fig trees under tropical field conditions. Egypt J Biol Pest Co. https://doi.org/10.1186/s41938-019-0133-9

Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept. Agric and Consumer Serv. Division of Plant Industry.

Ecoman Biotech. Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*). https: // www.en.ecomanbiotech.com / bio-nematicide. Accessed in March 2019

Gafur A, Swibawa IG. 2004. Methodes in Nematodes and Soil Microbe Research for Belowground Biodiversity Assessment. In: Susilo FX, Gafur A, Utomo M, Evizal R, Murwani S, Swibawa IG. Conservation and Sustainable Management of Below-Ground Biodiversity in Indonesia. Universitas Lampung. Indonesia

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.

Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.

Holland RJ, Williams KL, Khan A. 1999. Infection of *Meloidogyne javanica* by *Paecilomyces lilacinus*.Nematology 1(2): 131-139.

Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes. In: Luc M, Sikora RA, Bridge J (Eds.). Plant Parasitic Nematodes in Subtropical and Tropical Agriculture (2nd Edn). CAB International Publisher, UK.

Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. J Entomol Zool Stud 6(4): 1700-1704.

Jatala P. 1986. Biological control of plant parasitic nematodes. Ann Rev Phytopathol 24: 453-489.

Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode, *Pratylenchus thornei*. Tarim Bilimleri Dergisi. J Agric Sci 24: 323-328

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control 31: 346-352.

Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.

Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA & Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50(5):1800-1804.

Kumar S, Stecher G, Tamura K. 2016. MEGA:7 Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874

Kumar S, Rawat S. 2018. First report on the root-knot nematode *Meloidogyne enterolobii* (Yang and Eisenback, 1988) infecting guava (*Psidium guajava*) in Udham Singh Nagar of Uttarakhand, India. Int J Curr Microbiol App Sci 7(4): 1720-1724.

Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol Lett 321:141–149.

Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolat from mulet gut. Appl Environ Microbiol 57 (7): 1963-1968.

Paz BAO, Piedrahita OAG, Caycedo JL. 2015. In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root –knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. Bol Cient Mus Hist Nat 19 (2): 154-172.

Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J 95 (7-12): 415-417.

Razak AR, Lim TK. 1987. Occurrence of the root-knot nematode Meloidogyne incognita on guava in Malaysi. Pertanika. 10(3): 265-270

Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita.* Biocontrol Sci Technol 23 (12): 1469-1489.

Stanes T & Comapny Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. Accessed in June 2017.

Stirling GR. 2014. Biological Control of Plant-parasitic Nematodes (2nd Edn). CAB International Publisher. UK

Sundararaju P, Cannayane I. 2002. Production of nematode egg parasitic fungus, *Paecilomyces lilacinus,* on banana wastes and certain plant leaves. Indian J. Nematol 32 (2): 183-233.

Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017. Kendari. [Indonesian]

Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas. Padang. [Indonesian]

Wulandari C, Budiono P, Ekayani M. 2019. Impacts of the new decentralization Law 23/2014 to the implementation of community based forest management in Lampung Province, Indonesia. IOP Conf. Series: Earth and Environmental Science. 285 (2019) 012006. https://doi:10.1088/1755-1315/285/1/012006

475 Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG
476 24:141–144.

BIODIVERSITAS
Volume 21, Number 3, March 2020
Pages: xxxx

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d2103xx

Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia

I GEDE SWIBAWA[1,♥], YUYUN FITRIANA[1], SOLIKHIN[1], RADIX SUHARJO[1], F.X. SUSILO[1], EKA RANI[2], MEI SRI HARYANI[2], RACHMANSYAH A. WARDANA[2]

[1]Department of Plant Protection, Faculty of Agriculture, Universitas Lampung, Jl. Sumantri Brojonegoro No. 1, Bandar Lampung 35141, Lampung, Indonesia. Tel.: +62-721-787029, ♥email: igswibawa@yahoo.com; igede.swibawa@fp.unila.ac.id
[2]PT NTF Way Kambas, East Lampung 34375, Lampung, Indonesia

Manuscript received: 11 December 2019. Revision accepted: xxx February 2020.

Abstract. *Swibawa IG, Fitriana Y, Solikhin, Suharjo R, Susilo FX, Rani E, Haryani MS, Wardana RA. 2020. Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia. Biodiversitas 21: xxxx.* This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have a high pathogenic ability causing major losses in vegetable crops . The exploration of the fungi was carried out in 2016 and 2018 from Crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and NirAma, a commercial products which has been used for controlling *Meloydogyne* sp. in Indonesia. Identification was carried out based on morphological characteristics and molecular based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in a greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in five isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the discovered fungi was *Purpureocillium lilacinum* (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.). Based on thein vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4-100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Among of all the isolates tested, B01TG had the best ability to infect nematode eggs (99.5%), suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity compared to other isolates.

Keywords: Guava, pathogenicity test, *Purpureocillium lilacinum*, root-knot nematode

INTRODUCTION

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. (Swibawa et al. 2017). Three reported species of root-knot nematodes attacking guava are *Meloidogyne incognita* (Razak and Lim 1987), *M. Javanica* (Dawabah et al. 2019) and *M. enterolobii* (Kumar and Rawat 2018). According to Swibawa et al. (2017), all of the RKN attack rates is severe with a second-stage juvenile (J-2) population more than 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and continues to increas.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala 1986).

Purpureocillium. lilacinum has been used as a biological control agent for nematode in various countries and has shown positive results (Stirling 2014). Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al. 2006), root-knot nematodes in tomato plants (Singh et al. 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al. 2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be ableincreasing the amount and weight of fresh grains of wheat (Hernadez-Leal et al. 2016).

Purpureocillium lilacinum is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* using agar media and Sundararaju and Cannayane (2002) using rice, rice bran, and banana media, while Brand et al. (2009)

reproduced this fungus using solid form fermentation. *P. lilacinum* has been used as an active ingredient of bionematicides and is marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (Stanes and Company Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum*, such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hore et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to identify and reveal the pathogenicity of fungal egg parasites of RKN from the exploration at the guava plantation in Lampung.

MATERIALS AND METHODS

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice, June-October 2016 and April-September 2018, in Lampung Province, Indonesia. Exploration in 2016 was carried out in the Crystal guava plantations of PT. Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (4.9ºS and 105ºE) and PT. GGP PG4 East Lampung (05º00 '-05º15'S and 105º30'-105º45'E). Exploration was also carried out from NirAma, a commercial products which has been widely used for controlling *Meloidogyne* sp. in Indonesia. Exploration in 2018 was carried out in the Crystal guava plantations of PT. GGP PG4 (05º33'55.7964'S) and in Sinar Betung Village, Gunung Alip Sub-district, Tanggamus District (104º32 '21,516' E) (Figure 1).

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone at 20-40 cm of distance from the base of the stem with 0-20 cm of soil depth at four cardinal point following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD, Switzerland) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi (Figure 2) was separated for isolation purposes. Before isolation, the root samples containing the mass of RKN eggs were dipped in a 1% NaOCl solution for 30 seconds. The fungi infecting the egg mass of RKN were taken using a sterile needle and placed on the middle of the sterile Potato Sucrose Agar (PSA) medium that put on the sterile plastic petri dish (diameter 9 cm) under a sterile condition in Laminar Air Flow.

Identification of fungal egg parasites of root-knot nematodes

Morphological identification.

Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica DM500, Switzerland) at a magnification of 400-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett and Hunter 1998).

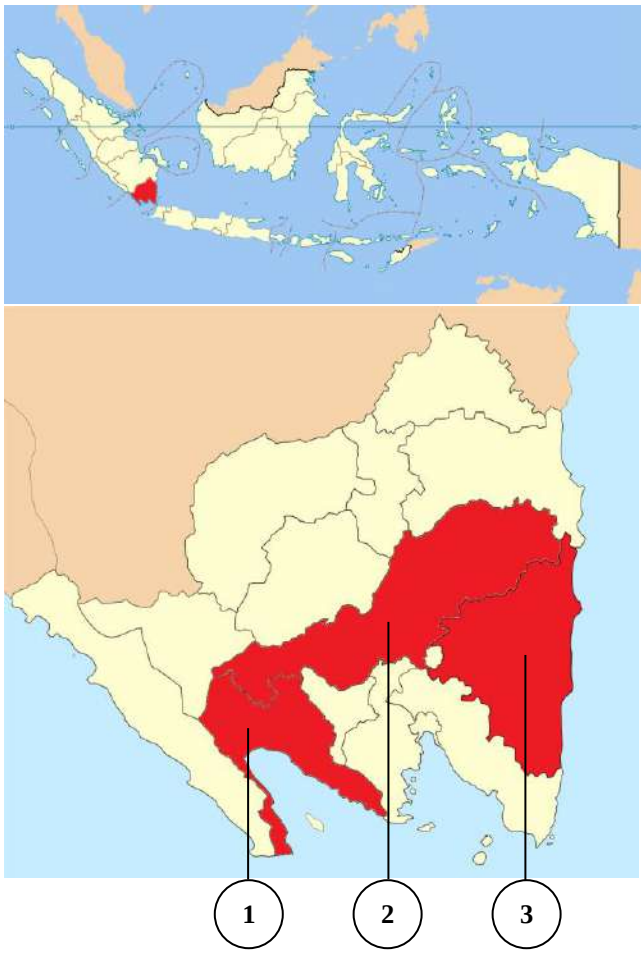


Figure 1. Sampling locations in Lampung Province, Indonesia: 1. Tanggamus, 2. Central Lampung, 3. East Lampung

Molecular identification

DNA extraction

The DNA extraction was carried out from fungal cultures aged three weeks. The fungi collected from the petri dish was suspended with 10 mL of sterile water in a 30 mL centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 mL of 70% chilled ethanol and centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed, and the pellet was added with 1 mL extraction buffer (0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water). The pellet was moved into a mortar and incubated at-38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. A total of 600 µL supernatant was then transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at-40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using a centrifuge (Microspin12, Biosan, Latvia).

PCR Amplification

Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction

The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hall 1999). The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al. 2016). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity test

In vitro test

The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates, including BioP that was isolated from NirAma, a commercial product obtained from PT. Great Giant Plantation (GGP) Plantation Group (PG) 1 Central Lampung in 2016, B4120X and B3010 that were isolated from PT. GGP PG4 East Lampung in 2016, B412G isolated from PT. GGP PG4 East Lampung in 2018 and B01TG that was isolated from Tanggamus in 2018. A drop (± 1 mL) of fungal spore suspension with a density of 10^8 spores/mL of potato water solution was added to a sterile petri dish with diameter of 9 cm. . Spore suspensions were spread evenly on sterile glass petri dishes, then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT. GGP PG4 East Lampung. An egg mass was taken every 12 hours to be observed under a Leica compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 3). The number of eggs infected with fungus is presented in percent.

Greenhouse test

Tomato plants cv. Victoria were used as test plants. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a polybag filled with 3 kg sterile soil. One day before the tomato planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck 1971). The intensity of root

damage was calculated using the formula of Ds = (∑vi x ni)/ (N x V) x 100%, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 mL of soil (Hooper et al. 2005). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa 2004). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

RESULTS AND DISCUSSION

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 in the Crystal guava plantations of PT GGP PG 4 East Lampung obtained two isolates. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT. GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. Onother isolate was obtained from PT. GGP PG 1, Terbanggi Besar, Central Lampung. The obtained isolates can be seen in Table 1.

Identification on the isolates of fungal parasites of nematode eggs

Identification based in morphological characteristics

Based on the identification, the mycelia and spores of the isolates obtained in this study were species of the genus *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony-forming air mycelia (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 4.A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 4.B).

Molecular identification

Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium lilacinum*. Based on the phylogenetic tree, the four isolates found were in the same group with *P. lilacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM-L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 5). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. At 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs in the range of 86.4% to 100%. The 100% infection was produced by B3010 and B412G isolates (Table 2).

The pathogenicity test in the greenhouse also showed relatively similar results to in vitro test. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table 3). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 mL of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010)-2571 (control) individuals/5 g roots and 615.2 (B412G)-1261.6 (control) individuals/300 mL of soil.

The application of *P. lilacinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lilacinum,* the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4).

Discussion

Purpureocillium lilacinum was originally described as *Paecilomyces lilacinus*. Based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al. (2011) moved *Paecilomyces lilacunus* into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Figure 2. The root gall containing egg mass of Root Knot Nematode (RKN) which was infected by the fungi. The red circle showed the fungi which was emerged

A **B**

Figure 3. Healthy (A) and infected (B) eggs of Root-knot nematode (RKN) *Meloidogyne* sp.

A **B**

Figure 4. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; Colony form (A); the shape of hyphae, conidiophore and conidia (B).

Table 1. The origin of isolates of fungal nematode egg parasites

Isolate name	Isolate origin	Location	Year of isolation
B4120X	Eggs of *Meloidogyne* spp.	PT. GGP PG 1, Central Lampung	2016
B3010	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	
BioP	Commercial products (NirAma)	Unknown	
B412G	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	2018
B01TG	Eggs of *Meloidogyne* spp.	Tanggamus	

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi Hours after infestation (HAI)				
	12	24	36	48	60
Control	0^{b}	0^{b}	0^{c}	0^{c}	0^{c}
BioP	11.3^{b}	11.3^{b}	56.8^{ab}	82.1^{ab}	93.6^{ab}
B4120X	1.1^{b}	1.1^{b}	70.9^{ab}	72.6^{b}	86.4^{b}
B3010	4.9^{b}	12.1^{b}	48.8^{ab}	82.7^{ab}	100.0^{a}
B412G	0.0^{b}	20.9^{b}	34.4^{bc}	89.8^{a}	100.0^{a}
B01TG	81.6^{a}	85.1^{a}	88.6^{a}	93.7^{a}	99.5^{a}
P>F	0.0001^{**}	0.0001^{**}	0.0107^{**}	$0.0001^{*^{*}}$	0.0001^{**}

Note: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 mL soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66
BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

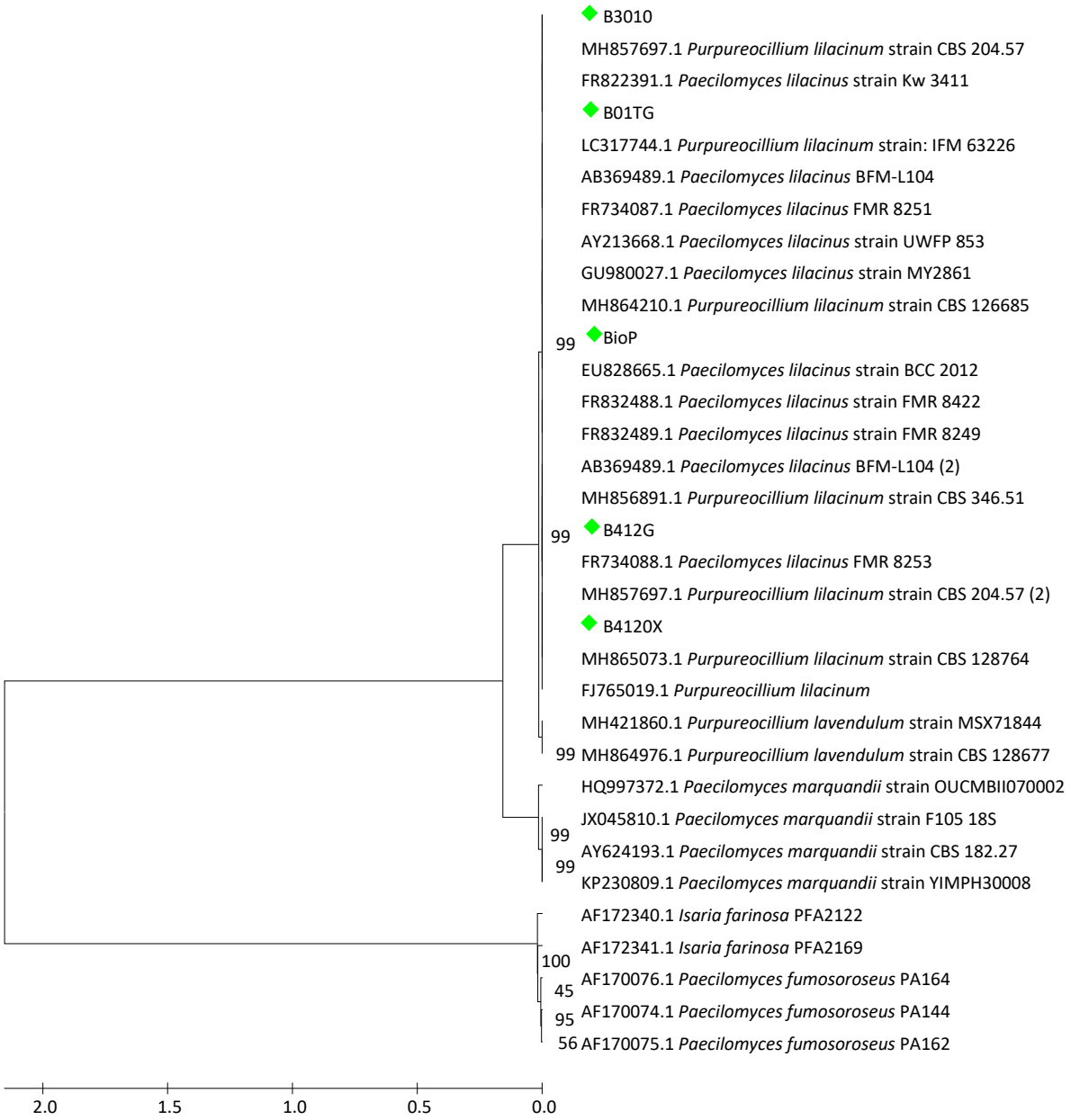


Figure 5. Phylogenetic tree based on the sequencing of ITS 1-ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). ♦ : The isolates of fungal nematode egg parasites discovered.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal of nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mentioned that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explained that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

Purpureocillium lilacinum isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and after that time, the number of eggs infected by the fungi increased. The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported that there was a variation in the pathogenicity of *P. lilacinum* isolates against *Meloidogyne* spp. The pathogenicity variation is thought to be influenced by the biophysical conditions of the environmental origin of the isolate. Isolates with high pathogenicity have the potential to be developed as bio nematicides. Fungal parasites of nematode egg are reported producing protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al. 2004; Bonants et al. 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita*. In line with the research of Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4-100%).

The research in the greenhouse also showed that the five *P. lilacinum* fungi had the ability to be pathogen of root-knot nematode. The application of fungal suspension in tomato plants infested with RKN can reduce J-2 nematode populations in soil and roots and decrease the root damage of tomato plants (unpublished data). Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

Based on the result, it can be concluded that the five fungal isolates used in this study were *P. lillacunum*. All the fungal isolates showed good capability to be used as biological control agent RKN, which were resulted more than 80% of eggs mass infection.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062/SP2H/LT/DRPM/ 2018 and Contract Pledge No. 393/UN26.21/PN/2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

REFERENCES

Barnett HL. 1969. Ilustrated Genera of Imperfect Fungsi. Second Edition. Burgers Publishing Company, USA.

Barnett HL, Hunter BB. 1998. Ilustrated Genera of Imperfect Fungi. 4th ed. Shop APS, USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal biological control agents through solid state fermentation: A case study on *Paecilomyces lilacinus* against root-knot nematodes. Micol Appl Intl 22 (1): 31-48.

Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Prot 124: 104874.

Dawabah AAM, Al-Yahya FA and Lafi HA. 2019. Integrated management of plant-parasitic nematodes on guava and fig trees under tropical field conditions. Egypt J Biol Pest Contr. DOI: 10.1186/s41938-019-0133-9

Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept Agric and Consumer Serv. Division of Plant Industry, Miami, FL.

Ecoman Biotech Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*). https: // www.en.ecomanbiotech.com/bio-nematicide. [March 2019]

Gafur A, Swibawa IG. 2004. Methodes in Nematodes and Soil Microbe Research for Belowground Biodiversity Assessment. In: Susilo FX, Gafur A, Utomo M, Evizal R, Murwani S, Swibawa IG. Conservation and Sustainable Management of Below-Ground Biodiversity in Indonesia. Universitas Lampung, Lampung. [Indonesian]

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.

Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.

Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes. In: Luc M, Sikora RA, Bridge J (eds.). Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. CAB International Publisher, Wallingford, UK.

Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. J Entomol Zool Stud 6 (4): 1700-1704.

Jatala P. 1986. Biological control of plant parasitic nematodes. Ann Rev Phytopathol 24: 453-489.

Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode, *Pratylenchus thornei*. Tarim Bilimleri Dergisi J Agric Sci 24: 323-328

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and

hatching of *Meloidogyne javanica* juveniles. Biol Control 31: 346-352.

Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.

Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA, Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50 (5):1800-1804.

Kumar S, Stecher G, Tamura K. 2016. MEGA:7 Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874

Kumar S, Rawat S. 2018. First report on the root-knot nematode *Meloidogyne enterolobii* (Yang and Eisenback, 1988) infecting guava (*Psidium guajava*) in Udham Singh Nagar of Uttarakhand, India. Int J Curr Microbiol App Sci 7 (4): 1720-1724.

Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol Lett 321:141-149.

Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolat from mulet gut. Appl Environ Microbiol 57 (7): 1963-1968.

Paz BAO, Piedrahita OAG, Caycedo JL. 2015. In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root-knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. Bol Cient Mus Hist Nat 19 (2): 154-172.

Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J 95 (7-12): 415-417.

Razak AR, Lim TK. 1987. Occurrence of the root-knot nematode Meloidogyne incognita on guava in Malaysia. Pertanika 10 (3): 265-270.

Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita.* Biocontrol Sci Technol 23 (12): 1469-1489.

Stanes T and Company Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. [June 2017].

Stirling GR. 2014. Biological Control of Plant-parasitic Nematodes (2nd Edn). CAB International Publisher, Wallingford, UK.

Sundararaju P, Cannayane I. 2002. Production of nematode egg parasitic fungus, *Paecilomyces lilacinus*, on banana wastes and certain plant leaves. Indian J Nematol 32 (2): 183-233.

Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017, Kendari. [Indonesian]

Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas, Padang. [Indonesian]

Wulandari C, Budiono P, Ekayani M. 2019. Impacts of the new decentralization Law 23/2014 to the implementation of community based forest management in Lampung Province, Indonesia. IOP Conf Ser: Earth Environ Sci 285: 012006. DOI: 10.1088/1755-1315/285/1/012006

Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG 24: 141-144.

BIODIVERSITAS
Volume 21, Number 3, March 2020
Pages: 1108-1115

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210334

Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia

I GEDE SWIBAWA[1,♥], YUYUN FITRIANA[1], SOLIKHIN[1], RADIX SUHARJO[1], F.X. SUSILO[1], EKA RANI[2], MEI SRI HARYANI[2], RACHMANSYAH A. WARDANA[2]

[1]Department of Plant Protection, Faculty of Agriculture, Universitas Lampung. Jl. Sumantri Brojonegoro No. 1, Bandar Lampung 35141, Lampung, Indonesia. Tel.: +62-721-787029, ♥email: igswibawa@yahoo.com; igede.swibawa@fp.unila.ac.id
[2]PT NTF Way Kambas, East Lampung 34375, Lampung, Indonesia

Manuscript received: 11 December 2019. Revision accepted: 18 February 2020.

Abstract. *Swibawa IG, Fitriana Y, Solikhin, Suharjo R, Susilo FX, Rani E, Haryani MS, Wardana RA. 2020. Morpho-molecular identification and pathogenicity test on fungal parasites of guava root-knot nematode eggs in Lampung, Indonesia. Biodiversitas 21: 1108-1115.* This study aimed to obtain and discover the identity of the species of fungal egg parasites of root-knot nematodes (RKN), which have a high pathogenic ability causing major losses in vegetable crops. The exploration of the fungi was carried out in 2016 and 2018 from Crystal guava plantations in East Lampung, Central Lampung, Tanggamus, and NirAma, a commercial product that has been used for controlling *Meloidogyne* sp. in Indonesia. Identification was carried out based on morphological characteristics and molecular-based gene sequential analysis of Intergenic Transcribed Spacer (ITS) 1 and ITS 4. A pathogenicity test was carried out in vitro and in a greenhouse using tomato plants as indicator plants. In the in vitro test, observations were made on the percentage of infected RKN eggs. The observations in the greenhouse test were carried out on RKN populations in the soil and roots of tomato plants, root damage (root knots), and damage intensity due to RKN infection. The exploration resulted in five isolates of fungal egg parasites of RKN from the guava plantations in East Lampung (2), Central Lampung (1), Tanggamus (1), and from the isolation results of commercial products (1). The isolates were given codes as B4120X (PT GGP PG1), B3010 (PT GGP PG4), B412G (PT GGP PG 4), B01TG (Tanggamus), and BioP (Commercial products). Based on their morphological characteristics, the isolates were classified into the genus of *Paecilomyces*. The results of molecular identification showed that the discovered fungi were *Purpureocillium lilacinum* (Thom.) Luangsa Ard. *(Syn. Paecilomyces lilacinus* (Thom.) Samson.). Based on the in vitro tests, the five fungal isolates were able to parasitize RKN eggs at 86.4-100%. In the greenhouse test, all isolates significantly suppressed nematode populations in the soil and tomato roots, inhibited the formation of root knots, and produced lower damage intensity compared to controls. Among all the isolates tested, B01TG had the best ability to infect nematode eggs (99.5%), suppressing the formation of root knots, nematode population in the soil and the roots of tomato plants, and the damage intensity compared to other isolates.

Keywords: Guava, pathogenicity test, *Purpureocillium lilacinum*, root-knot nematode

INTRODUCTION

Crystal guava plants in Lampung have been reported to be infected by root-knot nematode (RKN), *Meloidogyne* spp. (Swibawa et al. 2017). Three reported species of root-knot nematodes attacking guava are *Meloidogyne incognita* (Razak and Lim 1987), *M. Javanica* (Dawabah et al. 2019) and *M. enterolobii* (Kumar and Rawat 2018). According to Swibawa et al. (2017), all of the RKN attack rates is severe with a second-stage juvenile (J-2) population more than 1000 individuals per 5 g of roots. The problem of RKN in guava Crystal in Lampung is getting more serious because this nematode is difficult to control. Besides, its population is accumulative and continues to increase.

The use of chemical nematicides for controlling RKN in Crystal guava is not quite appropriate because the residues of the substances will harm the environment and can accumulate on the fruit, thus endangering the fresh fruit consumers. Therefore, the use of biological control agents such as fungal egg parasites of nematode for environmentally friendly and safe biological control programs for consumer health is highly recommended. One type of fungal agent for controlling nematode is *Purpureocillium lilacinum* (Thom.) Luangsa Ard. (Syn. *Paecilomyces lilacinus* (Thom) Samson). This fungus is also known as the fungal egg parasites of root-knot nematode, *Meloidogyne* (Jatala 1986).

Purpureocillium. lilacinum has been used as a biological control agent for nematode in various countries and has shown positive results (Stirling 2014). Besides effectively controlling root-knot nematodes, the fungus is also reported to be effective for controlling burrowing nematodes (*Radopholus similis*) in banana plants (Khan et al. 2006), root-knot nematodes in tomato plants (Singh et al. 2013), and root-lesion nematodes (*Pratylenchus thornei*) in wheat cropping in Turkey (Kepenekci et al. 2018). *P. lilacinum* is also reported to be able to promote plant growth. The application of *P. lilacinum* was proven to be able increasing the amount and weight of fresh grains of wheat (Hernadez-Leal et al. 2016).

Purpureocillium lilacinum is easy to find, isolate, and reproduce. Prabu et al. (2009) produced *P. lilacinum* using agar media and Sundararaju and Cannayane (2002) using rice, rice bran, and banana media, while Brand et al. (2009)

reproduced this fungus using solid form fermentation. *P. lilacinum* has been used as an active ingredient of bionematicides and is marketed under various trade names, such as Bio-Nematon, a bionematicide in the form of liquid and solid (Stanes and Company Limited 2017). Many commercial bionematicides contain active ingredients of *P. lilacinum*, such as Myco-Nematocure, Ukarsh Nematoz P, and Ecoman-R Bio-nematicide (Ecoman Biotch. Co., Ltd. 2014). Hore et al. (2018) mentioned that Bio-Nematon (*P. lilacinum* 1.15% WP) bionematicide was effective in controlling root-knot nematodes (*M. incognita*) in tomato plants.

The mass of RKN eggs attacking the guava plantations in Lampung was found to be parasitized by fungi. Swibawa et al. (2017) reported that the fungus that parasitized the mass of RKN eggs attacking the guava plantations in East Lampung was *P. lilacinum* with a parasitization rate of 16-26%. The discovery of this fungus indicates that there are fungal parasites of nematodes that have the potential to be explored and used as active ingredients of bionematicides. The application of indigenous (local biological agent) fungi to control nematodes is likely to be more effective than the use of exotic ones. More accurate identification to determine the identity of indigenous fungi is needed to study the biological characteristics in its use as a bionematicide active ingredient. This research aimed to identify and reveal the pathogenicity of fungal egg parasites of RKN from the exploration at the guava plantation in Lampung.

MATERIALS AND METHODS

Exploration of fungal egg parasites of root-knot nematodes

The exploration of fungal egg parasites of RKN was carried out twice, June-October 2016 and April-September 2018, in Lampung Province, Indonesia. Exploration in 2016 was carried out in the Crystal guava plantations of PT. Great Giant Plantation (GGP) Plantation Group (PG4) in Central Lampung (4.9ºS and 105ºE) and PT. GGP PG4 East Lampung (05º00 '-05º15'S and 105º30'-105º45'E). Exploration was also carried out from NirAma, a commercial product that has been widely used for controlling *Meloidogyne* sp. in Indonesia. Exploration in 2018 was carried out in the Crystal guava plantations of PT. GGP PG4 (05º33'55.7964'S) and in Sinar Betung Village, Gunung Alip Sub-district, Tanggamus District (104º32 '21,516' E) (Figure 1).

The samples of infected roots were collected from the Crystal guava plantations attacked by RKN. At each purposively selected plant, root samples were taken using a sucker in the root zone at 20-40 cm of distance from the base of the stem with 0-20 cm of soil depth at four cardinal points following the direction of the compass. Root samples from each plant were compiled and labeled for laboratory analysis. After being washed and dried, the roots were observed under a binocular stereomicroscope (Leica EZ4HD, Switzerland) at 40-60X magnification to find the mass of RKN eggs infected by fungi. The mass of RKN eggs infected by fungi (Figure 2) was separated for isolation purposes. Before isolation, the root samples containing the mass of RKN eggs were dipped in a 1% NaOCl solution for 30 seconds. The fungi infecting the egg mass of RKN were taken using a sterile needle and placed in the middle of the sterile Potato Sucrose Agar (PSA) medium that put on the sterile plastic petri dish (diameter 9 cm) under a sterile condition in Laminar Air Flow.

Identification of fungal egg parasites of root-knot nematodes

Morphological identification.

Fungal growth and development on PSA media were observed for morphological identification. The fungal morphological characteristics observed were the shape and color of the colony, as well as mycelium and spores. The shape and color of fungal colonies were observed directly, while the morphology of mycelia and spores was observed using a compound microscope (Leica DM500, Switzerland) at a magnification of 400-1000 X. The observed fungal morphological characteristics were confirmed with the fungi identification key (Barnett and Hunter 1998).

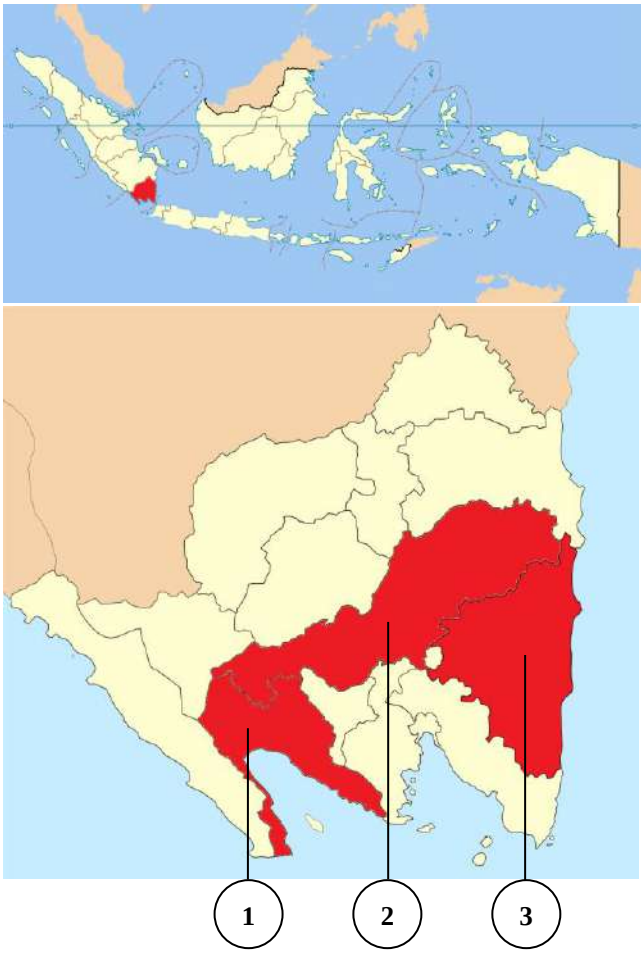


Figure 1. Sampling locations in Lampung Province, Indonesia: 1. Tanggamus, 2. Central Lampung, 3. East Lampung

Molecular identification

DNA extraction

The DNA extraction was carried out from fungal cultures aged three weeks. The fungi collected from the petri dish was suspended with 10 mL of sterile water in a 30 mL centrifuge tube, then centrifuged at 14,000 rpm for 10 minutes, then added with 1 mL of 70% chilled ethanol and centrifuged for 10 minutes at 14,000 rpm. The supernatant was removed, and the pellet was added with 1 mL extraction buffer (0.5 mL Tris HCl, 1 mL SDS 1% + 2.8 mL NaCl, 0.2 mL Mercaptho Ethanol, 2 mL EDTA, 3.5 mL sterile water). The pellet was moved into a mortar and incubated at-38ºC for 24 hours, then crushed until pulverized. As much as 500 µL pellet suspension was put into a 1.5 mL tube, and 400 µL of 2% cetyltrimethylammonium bromide (CTAB) was added, homogenized and heated at 65ºC for 1 hour using a water bath (Brookfield TC 550 MX-230, USA). After that, 500 µL of Phenol Chloroform Isoamyl (PCI) alcohol solution (25: 24: 1) was added, homogenized, and centrifuged at 14,000 rpm for 10 minutes. A total of 600 µL supernatant was then transferred to a new 1.5 mL tube, and 600 µL Chloroform Isoamyl Alcohol (24:1) was added, homogenized, and centrifuged again at 14,000 rpm for 10 minutes. A total of 400 µL supernatant was put into a new 1.5 mL tube, and 400 µL cold isopropanol was added, homogenized slowly by hand, and incubated at-40ºC for 20 minutes. The suspension was centrifuged again at a speed of 14,000 rpm for 15 minutes. The supernatant was removed, and the pellet obtained was then added with 500 µL of cool 70% ethanol. After that, it was then centrifuged again at a speed of 14,000 rpm for 5 minutes. The supernatant was removed, and the pellet obtained was incubated at room temperature for 24 hours to dry. After dry, the pellet was added with 50 µL 1x Tris-HCL EDTA (TE) pH 8.0 (Invitrogen). All centrifugation processes were performed using a centrifuge (Microspin12, Biosan, Latvia).

PCR Amplification

Amplification was carried out with a CFX Connect Real-Time PCR (Bio-RAD) machine by using 12.5 µL Master Mix (Red Mix) (Bioline), 1 µL ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3 '), 1 µL ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3 '), 2 µL DNA template and 8.5 µL sterile distilled water. DNA amplification using PCR consisted of one cycle of the initiation stage at a temperature of 95ºC for 5 minutes, 30 cycles of denaturation stages at a temperature of 95ºC for 1 minute, annealing at 44-56ºC for 1 minute, extension at 72ºC for 1 minute, and one cycle of elongation at 72ºC for 5 minutes. The PCR results were then electrophoresed using 0.5% agarose gel in 20 mL 1x buffer Tris-Boric Acid-EDTA (TBE) (Invitrogen) containing 1 µL Ethidium Bromide (EtBr 10 mg/mL). The electrophoresis was carried out using 1x TBE buffer at a voltage of 50 volts for 70 minutes. The results were visualized using DigiDoc UV transilluminator (UVP, USA).

Sequencing and phylogenetic tree construction

The PCR results were then sent to 1st Base Malaysia for the sequencing process. Sequencing results obtained were analyzed using the BioEdit program for windows ver. 7.2.6 (Hall 1999). The phylogenetic tree was constructed using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method with the MEGA 7 for Windows program (Kumar et al. 2016). The sequence data of ITS1 and ITS4 reference strains were taken from NCBI (https://www.ncbi.nlm.nih.gov/).

Pathogenicity test

In vitro test

The pathogenicity test of *P. lilacinum* isolates in vitro was carried out on *Meloidogyne* spp. eggs. The experiment was arranged in a randomized block design, consisting of six treatments and five blocks. The treatments were one control and five fungi isolates, including BioP that was isolated from NirAma, a commercial product obtained from PT. Great Giant Plantation (GGP) Plantation Group (PG) 1 Central Lampung in 2016, B4120X and B3010 that were isolated from PT. GGP PG4 East Lampung in 2016, B412G isolated from PT. GGP PG4 East Lampung in 2018 and B01TG that was isolated from Tanggamus in 2018. A drop (± 1 mL) of fungal spore suspension with a density of 10^8 spores/mL of potato water solution was added to a sterile petri dish with diameter of 9 cm. Spore suspensions were spread evenly on sterile glass petri dishes, then five masses of RKN eggs were placed in a circular position. RKN egg mass was obtained from PT. GGP PG4 East Lampung. An egg mass was taken every 12 hours to be observed under a Leica compound microscope at 400 X magnification. All eggs were chopped, including infected and healthy eggs (Figure 3). The number of eggs infected with fungus is presented in percent.

Greenhouse test

Tomato plants cv. Victoria was used as test plant. Tomato seeds were sown on a plastic tray measuring 31 cm x 24 cm x 4 cm containing sterile soil. After 14 days, they were then transplanted to a polybag filled with 3 kg sterile soil. One day before the tomato planted, the planting hole was inoculated with 1 mL of *P. lilacinum* spore suspension at a density of 10^8 spores/mL with the same type of treatment as in the in vitro pathogenicity test. One week after planting, all tomato plants were inoculated with 2000 root-knot nematode (*Meloidogyne* spp.) eggs/plant. Nematode eggs were obtained from the roots of the Crystal guava plants from PT GGP PG4, East Lampung showing symptoms of infection. Plant maintenance included watering, controlling weeds manually, and fertilizing using a mixture of N, P, K, and S with the composition of N (15%), P (15%) K (15%), and S (10%). Fertilizing was done three times, at 2, 5, and 8 weeks after replanting as much as 30g per polybag. At 98 days after planting, the soil and roots were taken for observation in the laboratory. Observations were made on the root damage, indicated by the formation of large galls or knots. The severity of the damage was determined using a scoring system based on the Zeck scale (0-10) (Zeck 1971). The intensity of root

damage was calculated using the formula of Ds = (∑vi x ni)/ (N x V) x 100%, where Ds = intensity of disease severity, vi = Zeck scale of root damage (0-10), ni = ith plant, N = number of plants measured, and V = the highest scale (10). Juvenile nematodes (J-2) were extracted using the modified Baerman method from 5 g roots randomly taken, while nematodes from the soil were extracted by stratified filtering methods and centrifuged with sugar solution from 300 mL of soil (Hooper et al. 2005). The J-2 nematode population was counted under a stereomicroscope (Leica EZ4HD, Singapore) at a magnification of 60 X (Gafur and Swibawa 2004). Data were analyzed using analysis of variance, and the separation of mean values was performed using the LSD test at a significance level of 5%.

RESULTS AND DISCUSSION

Isolates resulted from the exploration

The exploration of fungal parasites of RKN in 2016 in the Crystal guava plantations of PT GGP PG 4 East Lampung obtained two isolates. Exploration carried out in 2018 also found two isolates, each originating from the Crystal guava plantation of PT. GGP PG 4 East Lampung and the Crystal guava plantation in Tanggamus. Another isolate was obtained from PT. GGP PG 1, Terbanggi Besar, Central Lampung. The obtained isolates can be seen in Table 1.

Identification of the isolates of fungal parasites of nematode eggs

Identification based in morphological characteristics

Based on the identification, the mycelia and spores of the isolates obtained in this study were species of the genus *Paecilomyces*. The fungi grown on PSA media showed the characteristics of a colony-forming air mycelium (cotton) with a floccose-shaped edge. The color of the colony was initially white, then turned grayish violet after forming spores (Figure 4.A). At 1000 x magnification, the fungal hyphae seeped, and the conidiophores branched, forming phialides with round or oval conidia (Figure 4.B).

Molecular identification

Tracing of sequencing results using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) shows that the four isolates found had 99% similarity to *Purpureocillium lilacinum*. Based on the phylogenetic tree, the four isolates found were in the same group with *P. lilacinum* CBS 204.57 (Acc No. MH857697.1), Kw 3411 (Acc No. FR822391.1), IFM 63226 (Acc No. LC317744.1), BFM-L1048 (Acc No. AB369489.1), FMR 8251 (Acc No. FR734087.1), UWFP853 (Acc No. AY213668.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), MY2861 (Acc No. GU980027.1), CBS126685 (Acc No. MH864210), BCC2012 (Acc No. EU828665.1), FMR8422 (Acc No. FR832488.1), FMR8249 (Acc No. FR832489.1), CBS346.51 (Acc No. MH856891.1), FMR8253 (Acc No. FR734088.1) and CBS128764 (Acc No. MH865073.1) (Figure 5). This ensures that the four nematode egg parasite isolates found were *P. lillacinum.*

Pathogenicity of fungal nematode egg parasites discovered in this study

Five isolates of *P. lilacinum* tested proved to have high pathogenicity. At 60 hours after infestation (HAI), the five fungi were able to infect RKN (*Meloidogyne* spp.) eggs in the range of 86.4% to 100%. The 100% infection was produced by B3010 and B412G isolates (Table 2).

The pathogenicity test in the greenhouse also showed relatively similar results to in vitro test. The isolates of *P. lilacinum* were seen to be effective in controlling nematode populations. The population of J-2 nematodes in the roots of tomato plants in control treatments was higher than the population of nematodes in the roots of plants treated with the fungi (Table 3). Likewise, the J-2 nematode population in the soil was seen to be higher in control treatments than plants treated with *P. lilacinum* fungi. J-2 nematode populations in roots and soil appear to be influenced by the type of fungal isolate applied (Table 3). In plants treated with B01TG isolates, nematode populations reached 848.2 individuals/5 g roots and 424.8 individuals/300 mL of soil, which were lower than nematode populations in plants treated with other isolates whose populations were in the range of 1216 (B3010)-2571 (control) individuals/5 g roots and 615.2 (B412G)-1261.6 (control) individuals/300 mL of soil.

The application of *P. lilacinum* isolates was seen to be able to suppress the formation of root galls or knots. Root galls formed in control treatment reached a score of 7 from a maximum score of 10, while in the plants treated with *P. lilacinum*, the root galls formed were in the score of 2-4 of the maximum score of 10. The root damage intensity was also high in the control plants, reaching 66%, while in plants applied with the fungi, it was only around 18-40%. B01TG isolates were able to suppress the formation of root galls (score 2) and decrease the damage intensity (18%) (Table 4).

Discussion

Purpureocillium lilacinum was originally described as *Paecilomyces lilacinus*. Based on the 18sRNA sequence analysis results, Internal Transcribed Spacers (ITS) and partial Translation Elongation Factor 1-α (TEF), Luangsa-ard et al. (2011) moved *Paecilomyces lilacunus* into the genus of *Purpureocillium* and named *Purpureocilium lilacinum*. *P. lilacinum* is widely known as the fungus of the egg mass of the root-knot nematode (RKN) *Meloidogyne* spp. In the Crystal guava plantations in Lampung, the fungal egg parasites of RKN is easily found so that it has the potential to be developed into bionematicide for controlling RKN.

Figure 2. The root gall containing egg mass of Root Knot Nematode (RKN) which was infected by the fungi. The red circle showed the fungi which was emerged

A **B**

Figure 3. Healthy (A) and infected (B) eggs of Root-knot nematode (RKN) *Meloidogyne* sp.

A **B**

Figure 4. Fungal egg parasites of RKN discovered in the Crystal guava plantations of PT NTF; Colony form (A); the shape of hyphae, conidiophore and conidia (B).

Table 1. The origin of isolates of fungal nematode egg parasites

Isolate name	Isolate origin	Location	Year of isolation
B4120X	Eggs of *Meloidogyne* spp.	PT. GGP PG 1, Central Lampung	2016
B3010	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	
BioP	Commercial products (NirAma)	Unknown	
B412G	Rhizosphere of Crystal guava	PT. GGP PG4, East Lampung	2018
B01TG	Eggs of *Meloidogyne* spp.	Tanggamus	

Table 2. Percentage of the root-knot nematode eggs infected by each isolate of *P. lilacinum*.

Treatment of *P. lilacinum* isolates	% Root-knot nematode eggs infection by fungi Hours after infestation (HAI)				
	12	24	36	48	60
Control	0^{b}	0^{b}	0^{c}	0^{c}	0^{c}
BioP	11.3^{b}	11.3^{b}	56.8^{ab}	82.1^{ab}	93.6^{ab}
B4120X	1.1^{b}	1.1^{b}	70.9^{ab}	72.6^{b}	86.4^{b}
B3010	4.9^{b}	12.1^{b}	48.8^{ab}	82.7^{ab}	100.0^{a}
B412G	0.0^{b}	20.9^{b}	34.4^{bc}	89.8^{a}	100.0^{a}
B01TG	81.6^{a}	85.1^{a}	88.6^{a}	93.7^{a}	99.5^{a}
P>F	0.0001^{**}	0.0001^{**}	0.0107^{**}	0.0001^{**}	0.0001^{**}

Note: ** = significantly different based on F test; values followed by the same letters in the same column are not significantly different based on LSD test at 5%

Table 3. Population of root-knot nematodes in the soil and in the roots of tomato plants previously inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Nematode population	
	Indiv./5 g roots	Indiv. /300 mL soil
Control	2 571.0 a	1 261.6 a
BioP	1 951.2 b	899.2 b
B4120X	1 552.6 bc	813.6 b
B3010	1 216.0 cd	629.2 c
B412G	1 396.0 c	615.2 c
B01TG	848.2 d	424.8 d
P>F	0.0001	0.0001

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

Table 4. The root damage of tomato plants inoculated with root-knot nematodes and each of *P. lilacinum* isolates

Treatment of *P. lilacinum* isolates	Root damage	
	Zeck scale of root damage (0-10)	Damage intensity (%)
Control	7 a	66
BioP	4 b	40
B412X	4 b	36
B3010	3 c	30
B412G	3 c	30
B01TG	2 d	18

Note: Values followed by the same letters in the same column are not significantly different based on LSD test at 5%.

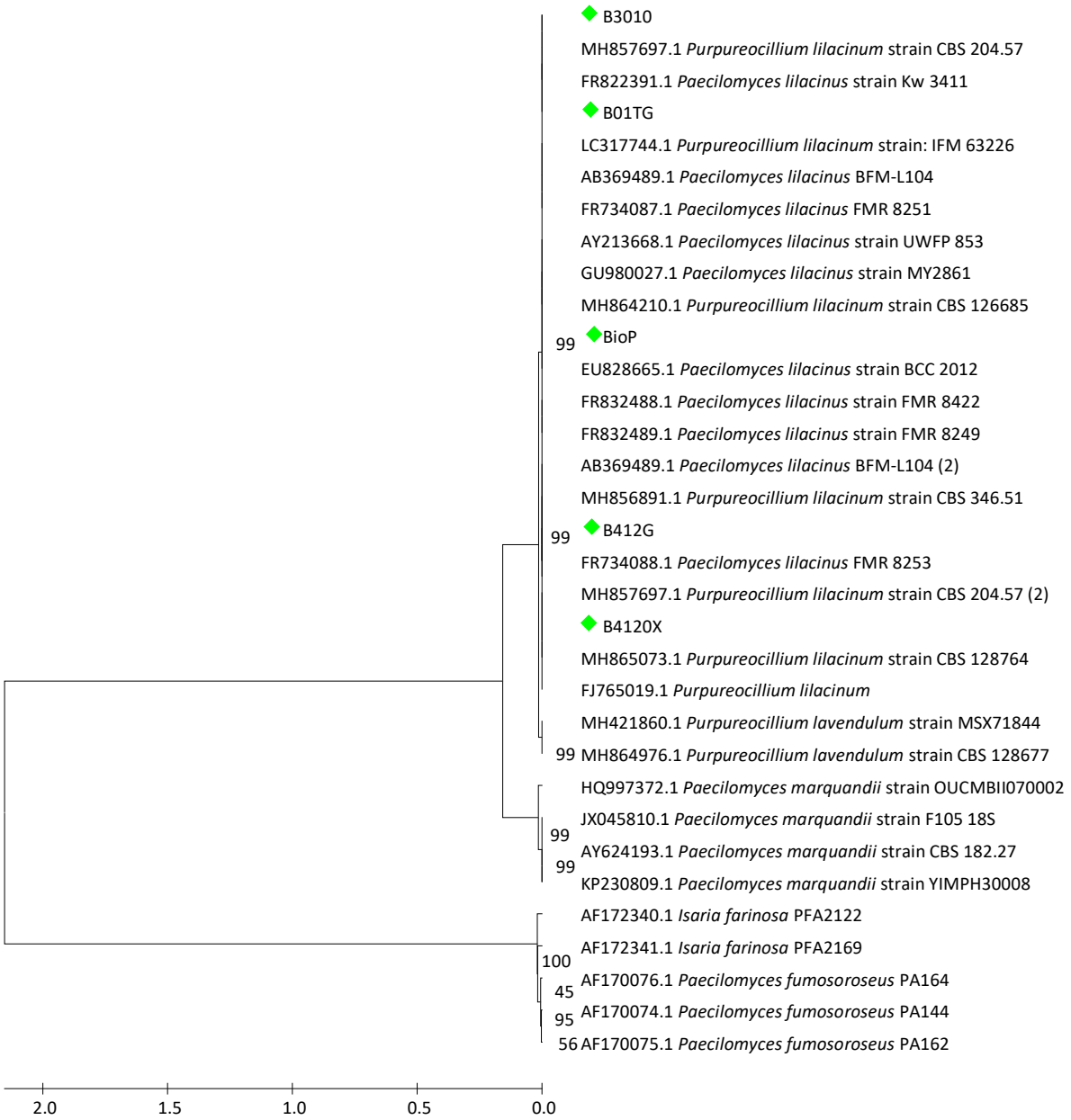


Figure 5. Phylogenetic tree based on the sequencing of ITS 1-ITS 4 using UPGMA method performed with Mega 7 program (Kumar et al. 2016). ♦ : The isolates of fungal nematode egg parasites discovered.

Based on the morphological characteristics and the analysis results of the ITS1 and ITS4 sequences, the isolates of fungal of nematode egg parasites found were *P. lilacinum*. Fungal colonies were seen forming air mycelia (cotton) with floccose-shaped edges. This characteristic is shared by the fungus in the *Paecilomyces* group (Barnett 1969). Mounfort and Rhodes (1991) stated that *P. lilacinum* has oval conidia of 2.5 µm long and 1.5 µm wide. Meanwhile, Esser and El-Gholl (1993) mentioned that *P. lilacinum* has a colony of light purple to dark purple, producing smooth to coarse conidia from a group of phialides growing from conidiophores. Khan et al. (2012) explained that *P. lilacinum* forms a white air mycelium, branched conidiophores arising from hyphae, and elliptic fusiform conidia, which are rarely round, single, or grouped.

Purpureocillium lilacinum isolated from the Crystal guava plantation in Lampung infected RKN eggs since 12 HAI, and after that time, the number of eggs infected by the fungi increased. The pathogenicity of the fungi isolates tested varied. For instance, the pathogenicity of B01TG isolates from Tanggamus was higher than that of other isolates. Winarto et al. (2017) reported that there was a variation in the pathogenicity of *P. lilacinum* isolates against *Meloidogyne* spp. The pathogenicity variation is thought to be influenced by the biophysical conditions of the environmental origin of the isolate. Isolates with high pathogenicity have the potential to be developed as bio nematicides. Fungal parasites of nematode egg are reported producing protease and chitinase enzymes to reduce the hatching of RKN eggs (Khan et al. 2004; Bonants et al. 1995). In vitro pathogenicity test conducted by Paz et al. (2015) showed that a mixture of *P. lilacinus* and *Pochonia clamidosporia* infected 80% of eggs and killed 90% of J-2 larvae of *Meloidogyne incognita*. In line with the research of Paz et al. (2005), the results of this study showed that the five *P. lillacinum* isolates had high infection ability. In 60 HAI, five fungal isolates were able to parasitize RKN eggs by more than 80% (86.4-100%).

The research in the greenhouse also showed that the five *P. lilacinum* fungi had the ability to be pathogen of root-knot nematode. The application of fungal suspension in tomato plants infected with RKN can reduce J-2 nematode populations in soil and roots and decrease the root damage of tomato plants (unpublished data). Dahlin et al. (2019) reported that the application of *P. lilacinum* combined with chemical nematicides could reduce the population of root-knot nematodes and root damage caused by root-knot nematodes.

Based on the result, it can be concluded that the five fungal isolates used in this study were *P. lillacunum*. All the fungal isolates showed good capability to be used as biological control agent RKN, which were resulted in more than 80% of egg mass infection.

ACKNOWLEDGEMENTS

The authors would like to thank the Ministry of Research and Higher Education for funding this study through Institutional National Strategy Grant Research Scheme based on Decree Number 062/SP2H/LT/DRPM/ 2018 and Contract Pledge No. 393/UN26.21/PN/2018. Gratitude is also expressed to the management of PT Great Giant Pineapple Lampung for their help and support in this research.

REFERENCES

Barnett HL. 1969. Illustrated Genera of Imperfect Fungsi. Second Edition. Burgers Publishing Company, USA.

Barnett HL, Hunter BB. 1998. Illustrated Genera of Imperfect Fungi. 4th ed. Shop APS, USA.

Bonants PJM, Fitter PLR, Thijs H, den Belder E, Waalwjck C, Henfling JWD. 1995. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 141: 775-784.

Brand D, Soccol CR, Sabu A, Roussos S. 2009. Production of fungal biological control agents through solid state fermentation: A case study on *Paecilomyces lilacinus* against root-knot nematodes. Micol Appl Intl 22 (1): 31-48.

Dahlin P, Eder R, Consoli E, Krauss j, Kiewnick S. 2019. Integrated control of *Meloidogyne incognita* in tomatoes using fluopyram and *Purpureocillium lilacinum* strain 251. Crop Prot 124: 104874.

Dawabah AAM, Al-Yahya FA, Lafi HA. 2019. Integrated management of plant-parasitic nematodes on guava and fig trees under tropical field conditions. Egypt J Biol Pest Contr. DOI: 10.1186/s41938-019-0133-9

Esser RP, El-Gholl NE. 1993. *Paecilomyces lilacinus* a fungus parasitizes nematode eggs. Nematology Circular, Fla. Dept Agric and Consumer Serv. Division of Plant Industry, Miami, FL.

Ecoman Biotech Co., Ltd. 2014. Ecoman® Bio-Nematicide (*Paecilomyces lilacinus*). https: // www.en.ecomanbiotech.com/bio-nematicide. [March 2019]

Gafur A, Swibawa IG. 2004. Methods in Nematodes and Soil Microbe Research for Belowground Biodiversity Assessment. In: Susilo FX, Gafur A, Utomo M, Evizal R, Murwani S, Swibawa IG. Conservation and Sustainable Management of Below-Ground Biodiversity in Indonesia. Universitas Lampung, Lampung. [Indonesian]

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98.

Hernadez-Leal T, Lopez-Lima D, Carrion G. 2016. Effect of the application of nematophagous fungus *Purpureocillium lilacinum* over nutrients availability on agricultural soil and yield of *Avena sativa*. Revista de la Facultad de Ciencias Agrarias 48 (2): 1-12.

Hooper DJ, Hallmann J, Subbotin SA. 2005. Methods for Extraction, Processing and Detection of Plant and Soil Nematodes. In: Luc M, Sikora RA, Bridge J (eds.). Plant Parasitic Nematodes in Subtropical and Tropical Agriculture. 2nd ed. CAB International Publisher, Wallingford, UK.

Hore J, Roy K, Maiti AK. 2018. Evaluation of Bio-Nematon (*Pupureocillium lilacinum* 1.15 WP) against root-knot nematode (*Meloidogyne incognita*) in tomato. J Entomol Zool Stud 6 (4): 1700-1704.

Jatala P. 1986. Biological control of plant parasitic nematodes. Ann Rev Phytopathol 24: 453-489.

Kepenekci I, Toktay H, Oksal E, Bozbuga R, Imren M. 2018. Effect of *Purpureocillium lilacinum* on root lesion nematode, *Pratylenchus thornei*. Tarim Bilimleri Dergisi J Agric Sci 24: 323-328

Khan A, Williams KL, Nevalainen HKM. 2004. Effects of *Paecilomyces lilacinus* protease and chitinase on the eggshell structures and hatching of *Meloidogyne javanica* juveniles. Biol Control 31: 346-352.

Khan A, Williams KL, Nevalainen HKM. 2006. Control of plant parasitic nematodes by *Paecilomyces lilacinus* on *Monacrosporium lusipagum* in pot trials. BioControl 51: 643-658.

Khan Z, Ahmad S, Al-Gimlas F, Al-Mutairi S, Josep L, Chandy R, Sutton DA, Guarro J. 2012. *Purpureocillium lilacinum* as a Cause of Cavitary Pulmonary Disease: a New Clinical Presentation and

Observations on Atypical Morphologic Characteristics of the Isolate. J Clin Microbiol 50 (5):1800-1804.

Kumar S, Stecher G, Tamura K. 2016. MEGA:7 Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874

Kumar S, Rawat S. 2018. First report on the root-knot nematode *Meloidogyne enterolobii* (Yang and Eisenback, 1988) infecting guava (*Psidium guajava*) in Udham Singh Nagar of Uttarakhand, India. Int J Curr Microbiol App Sci 7 (4): 1720-1724.

Luangsa-ard J, Houbraken J, van Doorn T, Hong SB, Borman AM, Hywel-Jones NL, Samson RA. 2011. *Purpureocillium*, a new genus for the medically important *Paecilomyces lilacinus*. FEMS Microbiol Lett 321:141-149.

Mounfort DO, Rhodes LL. 1991. Anaerobic growth and fermentation characteristic *Paecilomyces lilacinus* isolate from mulet gut. Appl Environ Microbiol 57 (7): 1963-1968.

Paz BAO, Piedrahita OAG, Caycedo JL. 2015. In vitro effect of *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al*. and *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams on the root-knot nematodes [*Meloidogyne incognita* (Kofoid & White) Chitwood and *Meloidogy nemayaguensis* Rammh & Hirschmann]. Bol Cient Mus Hist Nat 19 (2): 154-172.

Prabu S, Kumar S, Subramanian S. 2009. Mass production and commercial formulation of *Paecilomyces lilacinus*. Madras Agric J 95 (7-12): 415-417.

Razak AR, Lim TK. 1987. Occurrence of the root-knot nematode Meloidogyne incognita on guava in Malaysia. Pertanika 10 (3): 265-270.

Singh S, Panday RK, Goswami BK. 2013. Bio-control activity of *Purpureocillium lilacinum* strains in managing a root-knot disease of tomato caused by *Meloidogyne incognita*. Biocontrol Sci Technol 23 (12): 1469-1489.

Stanes T and Company Limited. 2017. Bio-Nematon. http://www.tstanes.com/products-bio-nematon.html. [June 2017].

Stirling GR. 2014. Biological Control of Plant-parasitic Nematodes (2nd Edn). CAB International Publisher, Wallingford, UK.

Sundararaju P, Cannayane I. 2002. Production of nematode egg parasitic fungus, *Paecilomyces lilacinus*, on banana wastes and certain plant leaves. Indian J Nematol 32 (2): 183-233.

Swibawa IG, Saputri ER, Yuliana E, Fitriana Y, Solikhin. 2017. Nematoda puru akar dan jamur parasitnya pada pertanaman jambu biji di Lampung. Seminar Nasional dan Kongres XIV Perhimpunan Fitopatologi Indonesia, 3-5 Oktober 2017, Kendari. [Indonesian]

Winarto, Darnetty, Liswarni Y. 2017. Potensi jamur *Paecilomyces* isolat lokal Sumatera Barat untuk pengendalian nematoda bengkak akar (*Meloidogyne* spp.) pada tanaman sayuran. Laporan Akhir Penelitian Unggulan Perguruan Tinggi. Universitas Andalas, Padang. [Indonesian]

Wulandari C, Budiono P, Ekayani M. 2019. Impacts of the new decentralization Law 23/2014 to the implementation of community based forest management in Lampung Province, Indonesia. IOP Conf Ser: Earth Environ Sci 285: 012006. DOI: 10.1088/1755-1315/285/1/012006

Zeck WM. 1971. A rating scheme for field evaluation of root-knot infestations. Pflanzenschutz Nachrichten Bayer AG 24: 141-144.