**Paecilomyces lilacinus** and **P. variotii** as a predator of nematode and trematode eggs

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**Abstract.** *Paecilomyces lilacinus* and *P. variotii* are molds that can control the parasitic worms for animals and plants. The molds have been used as biological control of plant nematodes and more commercialized, but for controlling animal trematode worm parasite has not been known. For the purposes of the experiments were tested in vitro the ability local isolates of *P. lilacinus* and *P. variotii* to reduce (predator) worm eggs of *Meloidogyne* sp and *Fasciola gigantica*. The tests carried out on the eggs of the two types of worms, by adding some eggs on *P. lilacinus* and *P. variotii* spores that have been inoculated in a petri dish. The experiments were performed during a specific time with the addition of isolates for a comparison test. The result of the test from this experiment was *P. lilacinus* is better than *P. variotii* to reduce types of those worms.

1. **Introduction**

One of the most harmful diseases of livestock is worm disease caused by *Fasciola* spp., and the disease is called fasciolosis. This disease affects the economy and the livestock industry, there is even a species of *Fasciola* sp is putatively zoonosis. In the ruminant livestock infected with fasciolosis have clinical symptoms of inhibited growth, decreased immunity, susceptible to other diseases, and found the worm eggs in the feces of animals that attacked by worms [1]. Fasciolosis is caused by *Fasciola hepatica* and *F. gigantica*. These worms include the trematode worms. Generally treated with anthelmintic, with the risk of the effect of resistance on worms and residues on livestock products.

Meanwhile rootworms infestation attacks on horticultural crops such as tomatoes, chili, potatoes, and others. The disease is detrimental because it lowers crop production with clinical symptoms of the affected plants yellow, easy to wilt, growth retardation and stunted, it is difficult to bear fruit. At the root of the infected are swelling that can be found that nematode worms. Generally, the nematode worm consist of *Meloidogyne* spp is classified as *M. incognita*, *M. javanica* and the others [2]. This root nematode attacks can generally be treated with anthelmintics anthelmintic with the risk of resistance effects on the worms and the residue on the plants. Root nematodes (*Meloidogyne* sp) cause considerable economic losses in agriculture and horticulture in the subtropics and tropics region [3].

Biological control is another option for dealing with worm disease without any risk of resistance and residue. Control of nematodes in farm animals and plant has been extensively researched and has been the stage of the application. whereas for this type of trematode worms have not been studied [4] [5].
Therefore it is necessary to explore more in the original mold Indonesia to be used as a biological control. While the use of biological control of plant nematode worms is already commonly known specifically genus Meloidogyne [6,7,8,9]. In accordance with the development and technological needs, it is estimated that 1 species of fungus that can potentially be used for two purposes. This biological control experiment for animals and plants may be the first time in Indonesia. *Paecilomyces lilacinus (Purpureum lilacinum)* local isolates were chosen because the literature based on mushrooms was allowed to be used, although it had to be investigated further [1]. So the purpose of this experiment is needed to do an *in vitro* test to prove the potential of *Paecilomyces* sp.

2. Materials

In this preliminary experiment to test the ability of local isolates of *Paecilomyces lilacinus* and *P. variotii* BCC (Balitvet Culture Collection) to reduce (eat) eggs *Fasciola gigantica* and *Meloidogyne* sp. We need *P. lilacinus* JCM (Japan Collection of Microorganisms) and local isolates, *Verticillium chlamydosporium* (local and JCM), *Duddingtonia flagrans*, *Trichoderma harzianum* and *T. asperelum* (local). The eggs of *Fasciola gigantica* and *Meloidogyne* sp from cattle and tomatoes.

3. Methods

3.1. Preparation of the test isolates

In this experiment, *P. lilacinus* and *P. variotii* BCC isolates tested were from the soil in West Java, Bandung, Bogor. The same treatment was carried out on *P. lilacinus* JCM (Japan Collection of Microorganisms) isolates, *Verticillium chlamydosporium* (local and JCM), *Duddingtonia flagrans*. Furthermore, *Trichoderma harzianum* and *T. asperelum* molds were given the same treatment as isolates on it. The mold was propagated in Petri containing Sabauraud Dextrose Agar (SDA) media and incubated for 5 days at room temperature (25-32°C). Spores/conidia of *P. lilacinus* and *P. variotii* were used, they were harvested by dredging and adding sterile water, then spores were calculated by hemocytometer.

3.2. Preparation egg of *Fasciola gigantica*

The eggs of the *F. gigantica* trematode worms to be tested are prepared from slaughterhouses. Egg collection according to [10]. The eggs are counted directly by pipetting eggs from a bottle containing an egg with a pipette and placing them on a special petri that has been given shading a rectangular box containing agar media. The eggs that have been obtained are stored in the refrigerator. Eggs can be used immediately when needed.

3.3. Preparation egg of *Meloidogyne* sp

*Meloidogyne* sp nematode worm eggs are prepared by collecting eggs. Eggs have been collected from the roots of tomato plants that have been infected with worms. In addition to roots, some soil samples are processed for eggs to be taken in the laboratory using the sedimentation-decantation method combined with centrifugation in a sugar solution of 454 g/l [11]. Furthermore, eggs and larvae are separated using the Baermann funnel technique [12]. The eggs are counted directly like *Fasciola gigantica*. The eggs obtained are stored in the refrigerator.

3.4. *In vitro* test is then performed as follows:

3.4.1. Assay of mold reduction against *F. gigantica* egg. Each of assay it was was prepared 100,000 spores of 5 isolates (*Paecilomyces lilacinus* (local and JCM), *Verticillium chlamydosporium* (JCM and local), and *P. variotii* (local), then mold inoculated into petri dish (Ø 5cm) which filled with 2% Bacto media, the mold was incubated for 24 hours then added with 100 eggs of *F. gigantica*. This experiment was conducted with 3 repetitions with daily observations for 4 days.
3.4.2. Assay of mold reduction against Meloidogyne sp egg. Each of assay were prepared 100,000 spores of 5 isolates (Paecilomyces lilacinus, P. variotii, Duddingtonia flagrans, Trichoderma harzianum, and T. asperelum, then mold inoculated into petri dish (Ø 5cm) which filled that 2% Bacto media, the mold was incubated for 24 hours then added with 100 eggs of Meloidogyne sp. These experiments were conducted with 3 repetitions with daily observations for 4 days.

4. Results and Discussion
Aside from the soil P. lilacinus isolates used in this experiment, the mold can be isolated from the feces and manure, this is in accordance with [13] which has successfully isolated P. lilacinus and V. chlamydosporium from feces and cattle manure. Meanwhile, the activity of P. variotii is not known. After in vitro assay the reduction of molds to eggs of F. gigantica and Meloidogyne sp have been obtained good results (Table 1). Untested in table 1. because the mold is not eligible to be tested as follows; 1. Molds are less able to adapt to grow. 2. The mold is considered too pathogenic to the host to whom it is treated. 3. Molds too difficult to apply. Among seven species of molds tested above have the ability to reduce nematode and trematode worm eggs. The local mold of P. lilacinus and P. variotii that has such potential, so that both Paecilomyces spp are feasible to be developed and potentially as reducing eggs of nematodes and trematodes. From 5 isolates tested Paecilomyces variotii local, P. lilacinus JCM and local, Verticillium chlamydosporium JCM and local. to its ability to reduce Fasciola gigantica eggs, 4 isolates potentially and 1 isolate (V. chlamydosporium local) no potential at all. All 4 of these isolates only P. lilacinus that can easily grow to adapt to tests that were conducted on its ability to grow [1].

Table 1. Type of mold and its ability to reduce eggs of Fasciola gigantica and Meloidogyne sp

| No. | Mould                          | Reduce egg of Meloidogyne sp (%) | Reduce egg of Fasciola gigantica (%) |
|-----|--------------------------------|---------------------------------|-------------------------------------|
| 1.  | Paecilomyces variotii (Bogor)  | 25,3                            | 100                                 |
| 2.  | Paecilomyces lilacinus (Bandung)| 67,9                            | 100                                 |
| 3.  | P. lilacinus JCM               | Untested                        | 100                                 |
| 4.  | Verticillium. chlamydosporium JCM | Untested                   | 100                                 |
| 5.  | V. chlamydosporium             | Untested                        | 0                                   |
| 6.  | Trichoderma harzianum          | 15,9                            | Untested                            |
| 7.  | Trichoderma asperelum          | 15,2                            | Untested                            |

While in vitro assay of the eggs of the nematode worms of Meloidogyne sp, of 5 isolates (Paecilomyces variotii, P. lilacinus, Duddingtonia flagrans, Trichoderma harzianum, and T. asperelum only D. flagrans which has no potential damage to eggs at all, and from to-4 isolates potentially only P. lilacinus that has the greatest potential in reducing eggs by 67.9%, meanwhile the potency of P. variotii only 25.1%. Meanwhile in the in vitro test the eggs of Meloidogyne sp nematode worm, from 5 isolates (P. variotii, P. lilacinus, D. flagrans, T. harzianum and T. asperelum only D. flagrans which did not have the potential to damage of egg, and from the 4th isolates potentially only P. lilacinus which has the greatest potential in reducing eggs by 67.9%, while P. variotii potential is only 25.1%. Although D. flagrans has been the potential to be a biological controller by trapping larvae, in damaging the worm eggs is not at all potential, this is in accordance with the opinion of [14,15] which states that D. flagrans target in reducing worm by trapping larvae, not its egg. Therefore it can be said that P. lilacinus in this preliminary experimental test has the potential to be a nematophagous and trematophagous mold. In observation in vitro assays of eggs F. gigantica, on the first day of 100 eggs, in the eggs can be seen for mycelial growth is still thin, the next day only 10 eggs that can be observed for many covered by mycelia. On the 4th day, all eggs were successfully overgrown by P. lilacinus (100% effective kill eggs) (Figure 1a).
Figure 1a. *Paecilomyces lilacinus* killed *Fasciola gigantica* egg

Figure 1b. *Paecilomyces lilacinus* killed *Meloidogyne* spp. egg

Figure 1. *Paecilomyces lilacinus* killed *Fasciola gigantica* egg (a) and *Meloidogyne* spp. egg (b) magnification 450x

In observation of *Meloidogyne* sp in vitro assay in 100 eggs, from observation for 4 days, *Meloidogyne* sp eggs are not all covered by mold (Figure 1b). This is in contrast to the observation on the egg of *F. gigantica* that all egg surfaces are overgrown by the mold tested. In observation of *Meloidogyne* sp eggs from 5 molds, only *P. lilacinus* which kill highest *Meloidogyne* sp eggs can kill as much as 67.9%, which means that not all the eggs were killed by the mold. Indeed, these observations are not 100 eggs were observed, while this is only able to observe the 50 eggs. This is due to limitations in observation in a microscope. But this can be analogized in the form of the percentage of the number of eggs killed by mold.

Why *P. lilacinus* potential as mold nematophagous and trematophagous, it will be described as follows; The mold of *P. lilacinus* is a filamentous fungus, having another name *Purpureocillium lilacinum* based on the isolation of rDNA-ITS Sequences DNA. Based on the phylogenetic analysis of *Paecilomyces* sp. Compared with the entomopathogenic species, *Paecilomyces* sp.. is classified in the Hypocreales [16,17]. Taxonomically *P. lilacinus* is classified in the fungi imperfecti or deutromycetes group. This mold has ascomata, ascus, and ascospore produced by teleomorphic species. The mold is generally saprobic, living in various habitats including cultivated or not like land, forest, grass, desert, and silt mud. molds can live in a wide temperature range. Some isolates of *P. lilacinus* can live in the temperature range 8-38°C, with an optimum growing temperature of 26-30°C. *P. lilacinus* also has a wide pH range tolerance and can grow on various substrates. *P. lilacinus* has been used as a biocontrol agent to control the destruction of root-knot nematodes (worms damage plant roots). Fungi can be entomopathogenic, mycoparasitic, saprophytic as nematophagous. Each isolate has a different pathogenicity level to plant nematode parasites. *P. lilacinus* produces enzymes such as serine proteases which are known to have biological activity against Meloidogyne hapla worm eggs. One strain of *P. lilacinus* is known to produce protease and chitinase, enzymes that can weaken the eggshell [18]. So it can be said that there is a lot of support from *P. lilacinus* biological properties as one of the molds for controlling worm eggs.

On macroscopic observation, the *P. lilacinus* colonies grow slowly in Sabauraud Dextrose Agar by 5-7 cm in diameter within 14 days at 25°C. His colony forms air mycelia (cotton) with a floccose fringe. At the beginning of growth is white, but when sprayed turns yellow, greenish yellow, brownish yellow, until violet. While the observation of the reverse side (below the petri dish) sometimes white or colorless but usually reddish brown according to age (Figure 2a). On microscopic observation, *P. lilacinus* have a thick and form mycelium conidiophores. Phialide located at the end of the spore formed in long chains. Phialide swelled on the basal and tapered to the neck. Vegetative hyphernys are smooth, hyaline, with a width of 2.5-4.0 μm. The conidia for arises from the hypha submerge at a length of 400-600 μm or from half the length of the hyphae in the air. Conidia is unicellular and chained, on a different chain.
of the fusiform ellipsoid, oval and smoothly walled (Figure 2b). This observation is in accordance with [19].

![Figure 2a. Paecilomyces lilacinus SGA media, Incubated 7 days Temp 25-34°C](image1)

![Figure 2b. Paecilomyces lilacinus magnification 400x staining Metylene Blue](image2)

**Figure 2. P. lilacinus. a) Macroscopic; b) Microscopic**

In the reduction of nematode eggs and trematodes, the mechanism of destroying the eggs is similar. It is just that both the nematode eggs and the trematodes certainly have a slightly different composition of egg composition, will be described as follows,

- **The mechanism of destruction trematode eggs**
  Before infecting trematode egg *P. lilacinus* grow to be spread on the surface of the egg, and later became more appress (sucking nutrients) to eggs of *Fasciola* sp. *P. lilacinus* produces simple appressoria in the eggshell as well after some hyphae grow on the surface after hyphae tissue is formed in eggs. The appearance of appressoria indicates that the egg has been or is being infected. In other cases, appressorium appears when the enlargement of the tip of the hypha tightly attaches to the eggshell. The adhesion between the appressorium and the surface of the egg must be strong enough to resist the opposite force resulting from the expansion of penetration by the tip of the hyphae. *P. lilacinus* have extracellular protease and chitinase enzyme. Chitin which has monopolysaccharide content is a solid structural component that is resistant to pressure. Protease will break down and remove the lipoprotein layer in the egg, then in the chitin layer hydrolysis by chitinase will occur, chitinase enzymes degrade chitin formed through the chitinolytic system by synergetic sequentially so that large vacuoles are seen, vitellin layer breaks and loss of integrity/density then there is damage to the structure of the egg. When hyphae have penetrated into the eggshell and then suck the contents of the egg, the hyphae will continue to grow until the entire egg cavity was filled with fungi. Then the mold destroys the egg quickly and then the egg dies, after that the hypha grows out of the empty eggshell and produces conidia which will then grow on the adjacent egg [1,18,20].

- **The mechanism of destruction nematode eggs**
  The vegetative hyphae enter the gelatin matrix of the root-knot nematode worm, then grow admission in the vulva or open the neck of the cyst from the nematode cyst, shortly after admission, the hyphae will form branches and grow on the surface of the eggshell. The tip of the hyphae swells to form an appressorium on the egg’s surface. Then the penetration is done by placing it under the appressorium then grow into the eggshell. Eggs are swollen and associated, penetration is continued on the vitellin layer of the eggs split into 3 parts, vacuoles in large quantities will appear, and the fat layer will almost disappear. Furthermore, the hyphae will fill the eggs and then appear to the egg surface resulting in the
first vegetative growth and conidia. Generally after 5 days almost all eggs in the infected mass. Juvenile in eggs can become infected when eggs are also invaded by molds as well as adult female worms can be infected [6].

The Opportunities of P. lilacinus as a nematophagous and trematophagous was considered by [1], but this year the initial study of in vitro testing was successfully carried out. The P. lilacinus experiment used as a biological controller for parasitic eggs of plant nematodes and animal trematodes can be the first to be carried out in Indonesia. Some literature states that P. lilacinus is a pathogen for humans, but is still under consideration because some isolates of Aspergillus sp which are considered dangerous for humans are still used as probiotics for livestock and consumed by humans. In addition, although the results of in vitro testing have not been satisfactory, such as in vitro P. lilacinus test is effective in reducing F. hepatica eggs [21], but when tested in the field does not always have the same effect, as in the isolation of P. lilacinus and V. chlamydosporium in the field to reduce ineffective F. gigantica worms [22]. In light of the foregoing preceding and keep in mind the bright prospects and opportunities to develop the potential of P. lilacinus as a predatory mold of the parasitic eggs of the nematode worms and trematodes. P. lilacinus research must be carried out in vitro on a large scale and many variants, when completed it must be continued in an in vivo test for livestock, and plants carried out in a greenhouse, then if successful and feasible followed by the application stage. The latter is generalized to farmers and farmers throughout Indonesia. Even though it takes a lot of time and money, it is a challenge to deal with future attacks of worm parasites.

The results of this initial experimental experiment (Table 1) showed that local P. lilacinus and P. variotii isolates have the potential to be one of the biological controllers of animal parasitic trematode worm eggs and plant nematode worm parasites. Paecilomyces spp. reduce the population of worms by killing/predators. P. lilacinus is better than P. variotii in reducing eggs of Meloidogyne spp and F. gigantica. Furthermore, further research is needed, namely in vitro tests in large numbers of worm eggs, in vivo tests on animals and plants in the field, applied and popularized.

5. Conclusion
Of the 7 molds of nematophagous and trematophagous isolates have been tested, only P. lilacinus is can be used as a biological control for Meloidogyne spp and F. gigantica.

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