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Molluscicidal Activity of Entomopathogenic Fungus, *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) against Golden Apple Snails, *Pomacea canaliculata* (Architaeniglossa: Ampullariidae)

(Aktiviti Moluskisid Kulat Entomopatogen, *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae) terhadap Siput Gondang Emas, *Pomacea canaliculata* (Architaeniglossa: Ampullariidae))

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ABSTRACT

Golden Apple Snails (GAS, *Pomacea canaliculata*) is known in Malaysia as one of the world's worst invasive pest species, wreaking havoc on paddy fields by lowering rice yield dramatically. Farmers now employ chemical molluscicides to eliminate GAS, but these are expensive and have serious health and environmental consequences. Biological control agent using entomopathogenic fungus is preferred over chemical molluscicides to control GAS because it is non-toxic, environmentally friendly and cost-effective. This study is aimed to investigate the molluscicidal activity of an entomopathogenic fungus, *Metarhizium anisopliae* towards GAS adults and eggs. *M. anisopliae* conidia were subsequently produced into four concentrations (3×10^4, 3×10^5, 3×10^6, and 3×10^7 conidia/mL) and tested against adults and eggs GAS. The median lethal time (LT_{50}) for the concentration of 3×10^7 took 5 and 3 days to kill 50% of GAS adults and inhibited 50% of eggs from hatching, respectively. Physiological analysis was also conducted on both GAS adults and eggs. The skin of infected individuals became white and opaque, while internal organs such as the pulmonary sac, digestive tract, and hepatopancreas were visibly destroyed. The colour of GAS eggs turned pale after *M. anisopliae* infestation and resulted in non-viable eggs. This research shows that *M. anisopliae* is an effective biological control agent for GAS and has the potential to be used as a targeted bio-molluscicide for GAS management.

Keywords: Biopesticide; entomopathogenic fungus; Golden Apple Snail; *Pomacea canaliculate;* sustainable agriculture

ABSTRAK

Siput Gondang Emas (GAS, *Pomacea canaliculata)* dikenali di Malaysia sebagai salah satu spesies haiwan perosak paling bahaya yang mendatangkan bencana kepada sawah-sawah padi dengan mengurangkan hasil tuaian padi secara dramatik. Pesawah kini menggunakan racun moluska kimia untuk menghapuskan GAS, akan tetapi cara ini agak mahal dan mendatangkan kesan yang serius kepada kesihatan dan persekitaran. Kaedah kawalan biologi menggunakan kulat entomopatogen lebih diutamakan berbanding racun moluska kimia bagi mengawal GAS kerana ia tidak toksik, mesra alam dan berkesan kos. Penyelidikan ini bertujuan untuk mengkaji aktiviti moluskisid bagi satu kulat entomopatogen, *Metarhizium anisopliae,* terhadap siput dewasa dan telur GAS. Konidia *M. anisoplia*e kemudiannya dihasilkan kepada empat kepekatan (3×10^4, 3×10^5, 3×10^6, dan 3×10^7 konidia/mL) dan diuji ke atas siput dewasa dan telur GAS. Tempoh masa maut median (LT_{50}) bagi kepekatan 3×10^7 untuk membunuh 50% siput dewasa dan menghalang 50% telur GAS daripada menetas, masing-masing mengambil masa 5 dan 3 hari. Analisis fisiologi juga telah dijalankan bagi kedua-dua siput dewasa dan telur GAS. Kulit individu yang dijangkiti menjadi putih dan legap, manakala organ dalaman seperti kantung pulmonari, saluran penghadaman dan hepatopankreas kelihatan hancur. Warna telur GAS bertukar menjadi pucat setelah dijangkiti *M. anisopliae* dan mengakibatkan telur-telur itu tidak berdaya hidup. Kajian ini menunjukkan bahawa *M. anisopliae* merupakan satu kaedah kawalan biologi yang berkesan untuk GAS dan berpotensi disasarkan sebagai bio-moluskisid untuk pengawalan GAS.

Kata kunci: Kulat entomopatogen; pertanian mampan; *Pomacea canaliculata;* racun perosak bio; Siput Gondang Emas

Introduction

Golden Apple Snails (GAS) or commonly known as *Siput Gondang Emas* is a large freshwater snail that is native to tropical and subtropical South America (Liu et al. 2018). In 1987, GAS (Mollusca: Ampullariidae: *Pomacea canaliculata*) was introduced in Malaysia as a food item and aquarium pet, but now it is one of the most serious pests that caused extreme damage to paddy fields (Nur Suraya, Noorshilawati & Rosminah 2017). Yahaya et al. (2017) reported that GAS is highly invasive and caused a drastic reduction in the production of rice in ASEAN countries, especially in Malaysia causing losses amounting to RM82 million in 2010. In addition, rice production is very important in Malaysia because rice is the main staple food needed by most Malaysians (Kasim et al. 2018). GAS is also categorized as one of the 100 world's worst invasive pest species because of its extensive reproductive capacity (Xu et al. 2017). Resh and Rosenberg (2015) reported that GAS can cause serious eutrophication which becomes a threat especially to local fishing, agriculture, and industries. Eutrophication usually happens when there is a decreasing number of macrophytes, such as pond weeds and hydrilla, which promotes the growth of algae. This is dangerous to aquatic organisms and cause massive death to them. GAS populations need to be controlled as GAS is one of the most serious pests that caused huge damage to the paddy crop. Current control strategies used by the farmers in Malaysia are mostly based on chemical molluscicides. Such molluscicides are like the illegal molluscicide containing fentin acetate, as well as molluscicides containing metaldehyde which are formulated in the forms of suspensions (Metasan 400) or granules (Esaro Snail Poison), whereas molluscicides containing niclosamide are usually in the form of emulsion (Kondor 25 EC), suspension concentrate (Mollus) or wettable powder (Bayluscide 70 WP) which deliver fast and effective responses (Noorshilawati, Nur Suraya & Siti Rosiyyah 2020: Rahim & Joshi 2019). However, the usual main active ingredients in molluscicides are metaldehyde or niclosamide are highly toxic and can cause water pollutions and toxicity to aquatic organisms (Schneiker et al. 2016). Moreover, recent findings show that chemical molluscicides are inefficient to control GAS since the GAS population show an increasing trend over the years (Hasyierah et al. 2012). This management approach has a detrimental influence on farmers' health and the environment, in addition to the high expenses of purchasing chemical molluscicides. Other green approaches such as using duck and fish as bio-control agents in controlling the population of GAS as one of their food sources have been reported (Abdullah & Reyhan 2017; Azmi et al. 2022; Teo & Hamsein 2017). However, this practice is less effective and impractical. The use of ducks as biological control is not efficient because the ducks may cause damage to young rice seedlings and burden the farmers as the farmers now must feed the ducks with commercial feeds (Liang et al. 2013). On the other hand, biological control using fish is much more difficult as enough water is needed to keep the fish alive (Su 2006).

There has been an increased interest in biological control agents in the last decade including fungal pathogens particularly entomopathogenic fungi (Azmi et al. 2022). Reddy, Zhao and Humber (2014) reported that entomopathogenic fungus, namely *Metarhizium anisopliae* (Hypocreales; Clavicipitaceae) is currently being used to control many agricultural and forest pests worldwide. *M. anisopliae* has been registered in the USA and other countries as a commercialized biopesticide agent. *M. anisopliae* has been proven effective in controlling insect pests and can kill the gastropods such as snails. It has been reported that *M. anisopliae* has ovicidal activity against aquatic planorbid snails, *Biomphalaria glabrata* (Duarte et al. 2015). A study by El-Sahn and Shairra (2012) found that *M. anisopliae* possessed a molluscicidal activity against garden snails, *Cornu aspersum*. However, the potential of *M. anisopliae* to infect adult GAS and their eggs has not yet been reported, particularly native isolate in Malaysia. Thus, a local isolate of *M. anisopliae* from Terengganu (Grace et al. 2017) was examined to see if it was pathogenic against different phases of GAS development. The findings of this study are intended to be employed as low-cost, environmentally friendly, and most significantly, safe for crops and human health alternative biocontrol agent for reducing GAS populations.

Materials and Methods

Collection of samples

The adults and eggs of *P. canaliculata* (GAS) were collected from an infested paddy field in Kampung Tualang, Manir, Kuala Terengganu, Terengganu, Malaysia (5.2987° N, 103.0667° E). Eggs were handpicked from stems and the leaves of the paddy plants whereas the adults were taken from the moist, shallow mud in the paddy field. All the adults with shell lengths ranging from 15-20 mm were collected and kept in aquarium tanks filled with fresh water and fed with mustard leaves

or *sawi* (*Brassica juncea*) once a day. There were 30 clutches of eggs that were collected carefully and kept in the plastic containers (dimension 120 mm top diameter × 65 mm height) containing distilled water at the bottom of the bottles with several holes on the top to ensure sufficient oxygen supply. Both samples were maintained under laboratory conditions at 25 ± 1 °C, 75 ± 5% of relative humidity and photoperiod of 12 h light: 12 h of darkness.

PREPARATION OF *M. anisopliae* CULTURE AND SOLID SUBSTRATE

The pure culture of *M. anisopliae* was provided by Grace et al. (2017). *M. anisopliae* were cultured and maintained based on Dotaona et al. (2017) and Ramle et al. (2016) with some modifications. The subcultured conidia were maintained at 28 ºC for about 14-20 days. 45 g of rice was washed thoroughly under running tap water and dried at room temperature for about 12 h. The rice was soaked in 45 mL distilled water, 9% yeast, and 0.02% chloramphenicol for 18 h. The rice was then cooked in a microwave for 4 min per flask, in casserole rice auto mode. The cooked rice flasks were autoclaved under 15 psi at 121 °C for 40 min and allowed to cool down to room temperature before being inoculated with *M. anisopliae*. The sterilized rice was inoculated by adding 10^8 conidia/mL of *M. anisopliae* and incubated at 28 ± 1 °C for 4 weeks.

HARVESTING OF CONIDIA AND PREPARATION OF CONIDIA SUSPENSION

After the incubation process was completed, the substrates covered with the conidia were collected and dried at room temperature for 1 week. The conidia then were separated from the substrates by using a two-nested sieve shaker with the mesh sizes of 2.0 mm to 500 µm. To prepare conidia suspension, 0.1 g conidia powder was dissolved in 1 mL of sterilized distilled water containing 0.02% Tween 80. Then, serial dilution with four dilution factors was carried out to form four different concentrations of conidia suspensions, which were 3×10^4, 3×10^5, 3×10^6, and 3×10^7 (conidia/mL). The concentration of conidia suspension was determined by using an improved Neubauer haemocytometer.

VIRULENCE OF *M. anisopliae* AGAINST GAS ADULTS AND EGGS

The method of pathogenicity test was done by following Leemon and Jonsson (2008). Adults of GAS were firstly sterilized with 40% ethanol and dipped three times in distilled water before infestation by the conidia suspensions. The GAS were sterilized to prevent any epibionts (such algae, ciliates, rotifers, and nematodes) on the shell and operculum of the GAS to affect the conidia of *M. anisopliae* which could affect the result of the experiment (Damborenea, Brusa & Negrete 2017). They were acclimatized in a plastic container with a perforated lid and fed with mustard before undergoing bioassay. The samples were then soaked in different conidia suspensions (10^4, 10^5, 10^6, and 10^7 conidia/mL) for 24 h each. For negative control, the samples were soaked in distilled water containing Tween 80, while for positive control samples were treated with 0.1 g of molluscicides, 'Racun Siput Berbutir' registered by Hwa Hong Trading Pty Ltd. This molluscicide consists of 5% metaldehyde as the active ingredient while the other 95% were inactive ingredients. Each replicate consists of 30 adults GAS and the experiments were repeated five times. Similarly, the eggs of GAS were sprayed with the prepared conidia suspensions (10^4, 10^5, 10^6, and 10^7 conidia/mL), positive and negative controls as mentioned for about 60 s, and then placed into the container individually (five clutches per replicate). Both the infected and the control GAS were incubated at room temperature, and the mortality of the samples was observed daily for 14 days. The bioassay took place in a controlled laboratory condition at 25 ± 1 °C and 75 ± 5% relative humidity. ANOVA (SPSS Version 25.0) was used to determine the differences in mortality rate between different treatments of conidia suspensions for each developmental stage of GAS. Tukey HSD Test was used to determine the significant difference within mean percentage of mortality rate and different treatments of conidia suspension. Median lethal time (LT_{50}) was used to determine the days that 50% of the GAS population were dead.

PHYSIOLOGICAL OBSERVATION AND FUNGAL ISOLATION

The body of dead adults was taken out from the shells and the comparison of physiological changes between infected and control GAS were observed using a stereomicroscope. The bodies were then cut into three parts which were head, middle body and tail. These body pieces were placed on a PDA and sealed for one to two weeks. The fungi were examined and inoculated onto a new PDA media. After an 18-h incubation period, the infected PDAs were stained with lactophenol cotton blue and identified under a microscope (Sun, Fuxa & Henderson 2003).

Results and Discussion

Pathogenicity of *M. anisopliae* against GAS adults and eggs

In this investigation, GAS adults were exposed to four different doses of *M. anisopliae* conidia suspension to assess their molluscicidal efficacy. The results showed that *M. anisopliae* was pathogenic to GAS adults under laboratory conditions with the different concentration having varying potency. This finding is in line with results obtained by El-Sahn and Shairra (2012) which reported that *M. anisopliae* possessed molluscicidal activity against garden snails, *Cornu aspersum.* The concentration of 3×10^7 conidia/mL treatment killed 50% of the GAS adults in 5.30 days compared to 3×10^4 conidia/mL treatment, which took 9.97 days to reach LT_{50} (Table 1).

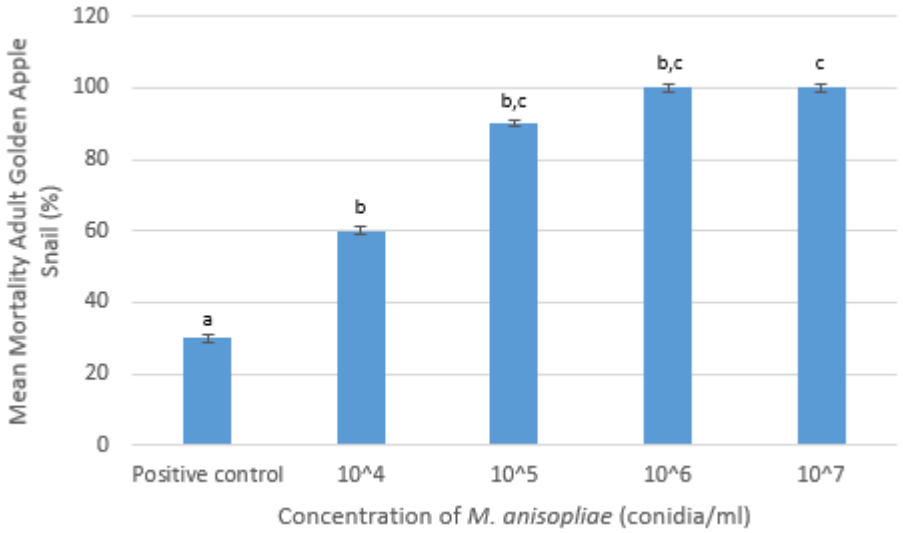
The colony and sporulation characteristics of *M. anisopliae* on PDA were observed. The mycelia mat observed was white and smooth. After the fifth day of incubation, dark green conidia mass with zonation was observed. The isolated fungus from infected GAS adults met with a description of *M. anisopliae* by Tulloch (1976). Faster and greater mortality rate were recorded from the treatments where higher conidia concentrations were applied than lower concentrations. This is perhaps because more conidia will help decrease the digestion time of the GAS cuticle since the presence of conidia can trigger the action of different enzymes simultaneously (Dai et al. 2011). Therefore, it is suggested that the concentration of 3×10^7 conidia/mL is the most effective and has the greatest molluscicidal activity in killing 50% of the GAS adult population. Interestingly, two concentration treatments, 3×10^6 and 3×10^7 (conidia/mL) could kill 100% of GAS adults on days 10 and 9, respectively, meanwhile with the same concentration, only 3% of adults garden snails (*C. aspersum*) was killed, as reported in results obtained by El-Sahn and Shairra (2012), indicating that *M. anisopliae* has greater molluscicidal activity against GAS compared to garden snails. All the conidia suspension treatments of *M. anisopliae* differed significantly in their virulence against adult GAS ($F = 11.328$, $df = 4, 69$; $p < 0.01$). Based on Figure 1, it is clearly showed that the increased mortality was closely related to the conidia concentration. The 3×10^7 conidia/mL concentration had the greatest mortality percentage of GAS adults and had considerably stronger molluscicidal action than the 3×10^4 conidia/mL concentration and the positive control.

In this study, *M. anisopliae* shows a promising result in killing GAS eggs also as the eggs were infective and virulent following treatment. GAS eggs recorded lower LT_{50} value as compared to GAS adults. This might be due to the surface of GAS eggs being different from GAS adults which resulted in different time taken for infection to occur. Despite this, the concentration of 3×10^7 conidia/mL treatment scored the most rapid reaction against the GAS eggs. Based on the lowest LT_{50} value, 50% of eggs were killed after 3.06 days of treatment (Table 1) (Figure 2). However, it was found that some eggs were tolerant to *M. anisopliae* even after 14 days of treatment aligning with results reported by Joshi et al. (2008) which found that older GAS egg masses were less susceptible to the saponin and the saponin only exhibited molluscicidal effects on fresh egg aged within 1-5 days old. Another study conducted by Duarte et al. (2015) on another species of aquatic planorbid snail, *Biomphalaria glabrata* eggs, concluded that although *M. anisopliae* has the ability to kill the eggs of *B. glabrata,* it did not completely inhibit eclosions of the eggs. In this study, conidia suspensions with concentrations of 10^4, 10^5, 10^6 and 10^7 all achieved 100% mortality rate on day 7, 6, 5, and 4, respectively. The eclosion of larvae from eggs treated with conidia was inhibited by the presence of *M. anisopliae* on egg surface with development of fungal metabolites.

TABLE 1. LT_{50} of adults and eggs of golden apple snails (GAS) treated with different concentration of *M. anisopliae* conidia suspensions

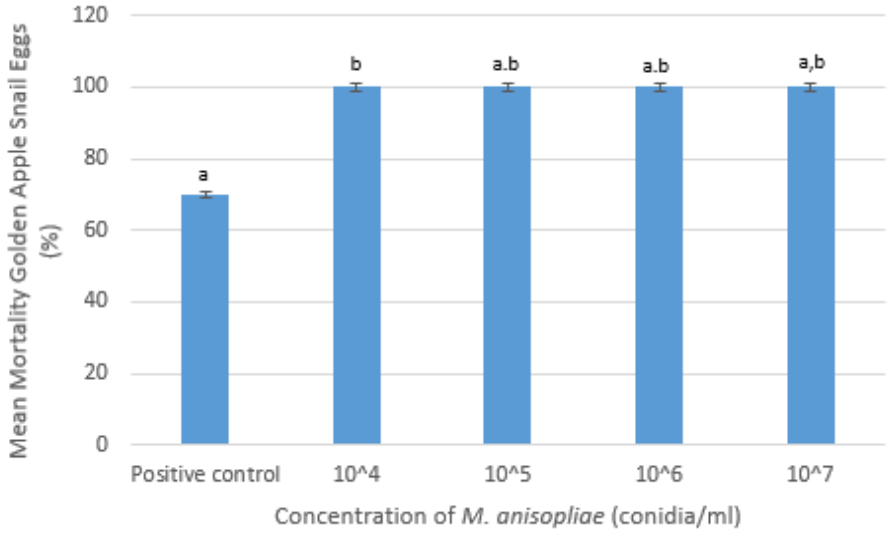
Concentration of *M. anisopliae* (conidia/mL)	LT_{50} (DAT)	
	Adult	Egg
3×10^4	9.96_{a}	6.38_{a}
3×10^5	$7.72_{a,b}$	$5.35_{a,b}$
3×10^6	$6.44_{a,b}$	$4.99_{a,b}$
3×10^7	5.30_{b}	3.06_{b}

DAT = days after treatment, followed by different letters which represent significant difference at $p < 0.05$ (Tukey HSD Test)



Same letter in the graph indicates no significant differences at $p < 0.05$ (Tukey HSD Test)

FIGURE 1. The average percentage of total mortality of adult golden apple snails treated with different concentrations of *M. anisopliae* after 14 days of treatment



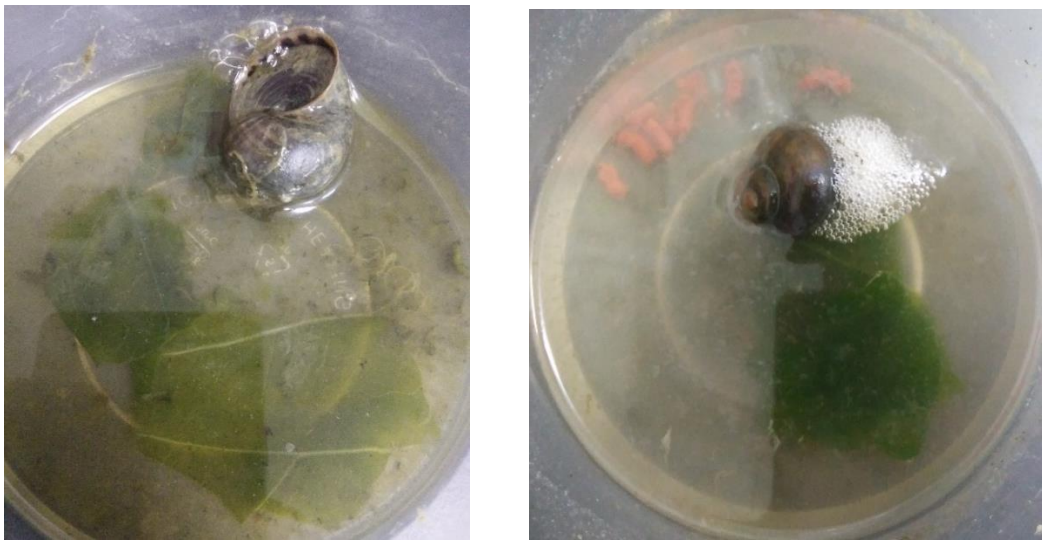
Note: Same letter in the graph indicates no significant differences at $p < 0.05$ (Tukey HSD Test)

FIGURE 2. The average percentage of total mortality of golden apple snail eggs treated with different concentrations of *M. anisopliae* after 14 days of treatment

PHYSIOLOGICAL OBSERVATIONS ON GAS ADULTS AND EGGS

The physiological observation was conducted to describe the signs and symptoms of infected GAS. The presence of bubbles was a symptom of the dead in GAS adults when exposed to molluscicide. Based on Figure 3(a), there was no bubble observed from the operculum of dead adults in the negative control treatment. In contrast, many bubbles were produced from the operculum of adults GAS treated with *M. anisopliae* and positive control (Figure 3(b)). Moreover, there were physiological changes observed on GAS adults which resulted in the changing of skin colours where the skin colour of GAS turned from translucent colour to white opaque which after GAS adults were exposed to the *M. anisopliae* within 5 days. The negative control GAS adults had almost translucent skin colour (Figure 4(a)), while *M. anisopliae* infected GAS adults had white and opaque skin colour (Figure 4(b)). The colour is thought to have changed after GAS adults were dead. Thus, it shows that *M. anisopliae* might contain active compounds that altered the skin colour of the GAS, which needs further investigation.

Aside from that, there were also obvious signs of physiological damages to the organs of infected GAS. The organs such as pulmonary sac, hepatopancreas and digestive part of infected GAS were destructed, disintegrated and had visibly burst however, the organs of GAS adults control group that had negative treatment did not burst, maintained the original shape and colour and were fixed on the proper position in the body of GAS. This result is in line with the results obtained by Gohar et al. (2014) where the study concluded that the fruits, leaves and bark extracts of *Callistemon viminalis* showed great histopathological signs to the digestive tract of the *Biomphalaria alexandrina* snails. They found that *C. viminalis* fruit extract resulted in the destruction in the follicular membrane, and the mature ovum showed losing the nucleolus. Hence, they believed that the plant extracts have molluscicidal activity against *B. alexandrina*. In addition, previous study by Shen et al. (2018) found that basal lamina and epithelial cell of the digestive cell, and digestive gland tubules structure of hepatopancreas of GAS were lysed, denatured, and incomplete or became necrotic which positively correlated with dose in *Solidago canadensis* extracts. Ke et al. (2017) reported that the alkaloid component in plume poppy, *Macleaya cordata* leaves potent great molluscicidal activity against *Oncomelania hupensis* snails. They found that hepatic failure might have occurred in the snail hepatopancreas after being treated

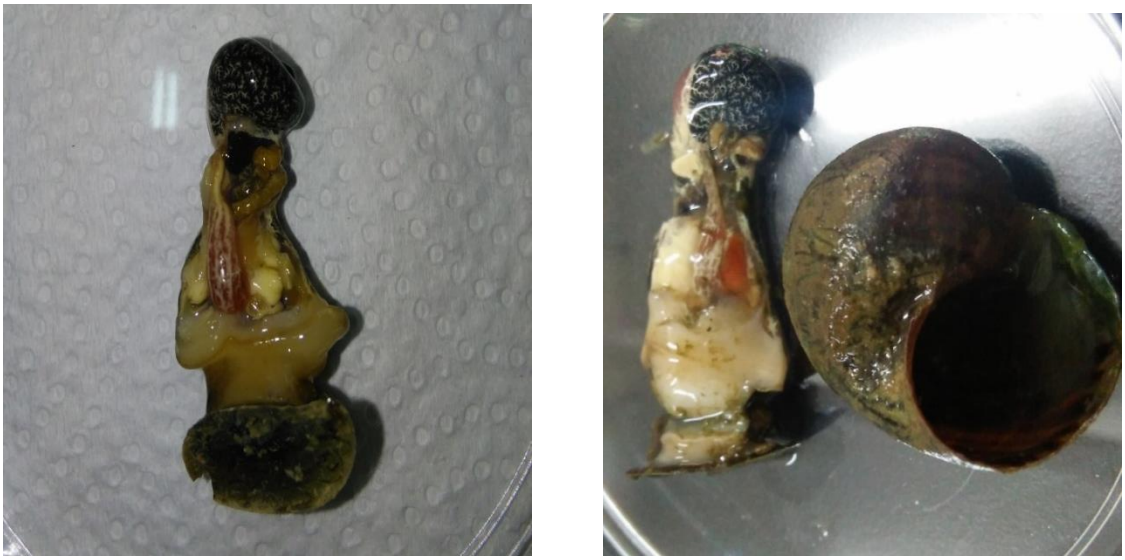


(a) (b)

FIGURE 3. Sign and symptoms of infected golden apple snail (GAS) adults. Negative control of GAS adult (a) and infected of GAS adult (b)

at a high concentration of *Macleaya cordata* extract. The present study found that the hepatopancreas of GAS degenerated after being treated with *M. anisopliae* which is similar to Ke et al. (2017) observation. As reported by Ke et al. (2017), the physiological toxic mechanisms of alkaloid component against snails might disturb normal physiological metabolism of snails such as inhibiting oxidative phosphorylation and protein synthesis in the respiratory chain, and weakening the detoxification ability of hepatopancreas, which caused higher in mortality rate of the snails. To summarize, all of these physiological changes to organs of GAS recorded are in line with results obtained by prior studies, proving positive molluscicidal activity of *M. anisopliae* against GAS by possibly targeting its digestive part, pulmonary sac and hepatopancreas. Destruction of the membrane of the organs and decolouration of the skin were the physiological sign detected after treatment with different concentrations of *M. anisopliae*.

The actual colour of GAS eggs are bright pink. However, the present study recorded that the infection of *M. anisopliae* has changed the colour of GAS eggs from bright pink colour up to pale pink after 10 days of infection. According to the findings on GAS eggs, the higher the concentration of *M. anisopliae*, the higher the prevalence of non-viable eggs. GAS eggs with a bright pink colour indicate viable eggs (Figure 5(a)), while GAS eggs with a light pink colour indicate non-viable eggs (Figures 5(b)). Discoloration of egg shell also a symptom of infection by *M. anisopliae* which is consistent with this finding. After discoloration occurred, the fungal slowly started to grow on the surface of GAS eggs. It took more than 14 days for the fungal growth to be seen clearly on the eggs shell.



(a) (b)

FIGURE 4. Physiological observation of internal organs between negative control (a) and infected (b) GAS adults

Negative control (a), positive control (b), infected GAS eggs after 3 days (c), 7 days (d) and 10 days (e) of infection

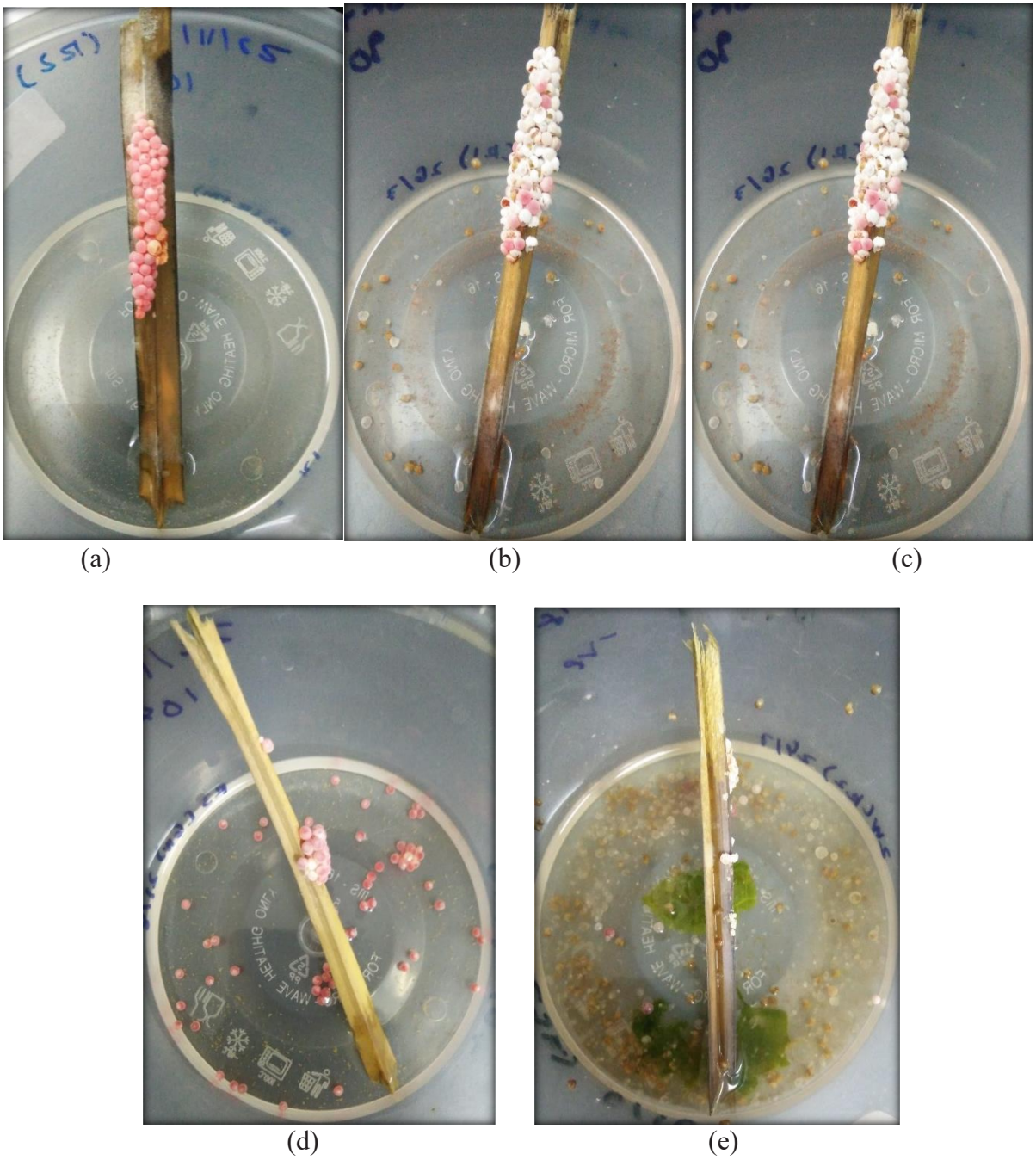


FIGURE 5. Signs and symptoms of infected golden apple snail (GAS) eggs

CONCLUSIONS

Results obtained from this study clearly illustrated that the entomopathogenic fungus, *M. anisopliae* caused mortality in adults and eggs of GAS which showed that this invasive rice species are susceptible to the infection by *M. anisopliae*. The conidia suspensions of *M. anisopliae* exhibited pathogenicity with 100% mortality within 14 days of post-infection for both adults and eggs of GAS at optimum concentration of 3×10^7 conidia/mL. Therefore, the use of entomopathogenic

fungus, *M. anisopliae* might be a useful component in the Integrated Pest Management (IPM) program against GAS. Further study should be carried out especially on the technical feasibility of the application of *M. anisoplaie* as a molluscicide for GAS in the field which requires a large scale of conidia suspension of *M. anisopliae.*

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