Advanced and Sustainable Technologies
ASET
E-ISSN 2976-2294
Volume 4, No 2, December 2025 [363-370]

Isolated Fungi as Bio-Control Agents in Disturbing Growth of Golden Apple Snail, *Pomacea canaliculata*

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Received 3 September 2025, Revised 28 September 2025, Accepted 16 October 2025

ABSTRACT

The golden apple snail, or scientifically known as Pomacea canaliculata, is a significant threat to paddy farming in many countries, including Malaysia. This study focuses on producing green biopesticides from microbial by-products with a strong mode of action against these snails. To achieve this, nine microorganisms were isolated, screened, characterized and molecularly identified. The isolates were classified according to their morphological and also reproductive characteristics. Findings show that isolates KP1 to KP5 are fungi with sporangiospores, isolates KP6 to KP8 are fungi with conidiospores, while KP9 is a fungus with an arthrospore. Further screening showed that non-autoclaved filtrates of KP3 and KP8 had the highest molluscicidal activity at 47% and 50% respectively. Two potential fungi with molluscicidal activity were identified as Talaromyces stipitatus and Aspergillus awamori.

Keywords: Golden Apple Snail, Isolation, Microorganism, Pathogenic Fungi.

1. INTRODUCTION

Pomacea canaliculata or golden apple snail (GAS) is one of the most important pests in paddy cultivation in Malaysia, as in other Southeast Asian countries. This nocturnal pest is consumed on mostly soft-stem crops such as paddy seedlings up to one month of age. The damage caused by this snail depends on their size and population [1]. Paddy seedlings of more than thirty days old are mostly targeted, and this caused problems with direct seeding, where the damaged seedlings from direct seeding need to be replanted or transplanted, which is costly and also time-consuming. Up to date, GAS is now widely spread in major rice-growing areas in Malaysia, such as Perlis, Kedah, Selangor, Pulau Pinang, and Kelantan. There are several factors that promote invasions of this snail, such as a high reproduction rate and adaptability to new environments [2-4].

Various control measure was adopted in controlling damages caused by snails, especially in paddy fields through chemical, physical and biological methods. The chemical method is the most favorable approach due to its fast killing action. However, certain chemicals, such as niclosamide and methaldehyde, can cause non-target toxicity, environmental pollution, produce pesticide residues and develop drug resistance [5-6]. Physical approaches applied, such as hand-picking of snails, destroying egg-clusters, transplanting, installing a screen in water inlets, field drainage and others were also reported [7-8]. However, this method is labor-intensive, costly and also less effective. Ducks, fish and soft-shelled turtles are common predators used in a biological approach to control snail population in the field [9-12]. Plant extracts are commonly used in the study of

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biopesticides against GAS. In their study on molluscicidal effect of green tea seed, red spider lily, tobacco and sophora, Cho et al. [14] stated that organic materials from plant powders could help control GAS in an eco-friendly manner.

These control approaches can partially reduce snail infestations and crop damage. However, they only help in minimizing the problems; thus, further research and alternative measures are necessary to explore and develop more effective solutions. Biological approaches, such as the application of microorganisms, can be used, which are more environmental friendly and cost-effective. Thus, this research aims to isolate microorganisms from dead GAS, characterize and identify the microorganisms, and utilize them as a bio-control agent against GAS.

2. MATERIAL AND METHODS

Sampling of the snails was carried out during the dry and off-season in the irrigation stream in Simpang Empat and Kuala Perlis, in the state of Perlis, in the northern region of Malaysia. Dead snails collected show symptoms of swollen flesh and floated on the water's surface. For rearing purposes, snails were randomly scooped from various spots of the canals and then placed in a basin containing water taken from the canals. The snails were immediately brought back to the laboratory of the Faculty of Chemical Engineering & Technology, UniMAP, Jejawi. All snails were cleaned to remove dirt. Snails of 2.93 ± 0.36 cm (length) and 6.40 ± 1.89 g (weight) were selected from the collection and then transferred into the growth tank. A sufficient amount of fresh water, ferns and fish pellets as a food source for the snails were fed twice daily. The snails were acclimatized in lab conditions for a week following the method by Akinpelu et al. [15].

2.1 Isolation of Fungi

Dead snails were collected from the irrigation canals and kept in a cold room of 4°C prior to use. Microbial isolation was conducted by using the dead flesh of the snails. Using a sterile inoculating loop, a loopful of sample was spread onto potato dextrose agar, performed in three replicates. Microorganisms grown on potato dextrose agar (for fungi growth) were left in an incubator at 30˚C for a week. Growth of microorganism on both agar was subcultured to obtain a pure strain. Using a sterile inoculating loop, a loopful of isolate from single colonies on potato dextrose agar was spread onto a new agar plate and left in the incubator at 30˚C for a week. Strain growth was observed, and subculture was performed until pure colonies were obtained. Fungal isolates were grown on slant and kept at 4˚C.

2.2 Characterization of Fungi

The method of staining was done according to Prescott, Harley, and Klein [16]. A loopful of fungi was inoculated on new agar using a sterile inoculating loop and left to grow for four days at 30°C. After four days, using a sterile loop, a small portion of fungi was fixed onto the glass slides using simple staining with methylene blue. Size, shape, and arrangement of the microorganism were observed under the light microscope.

2.3 Screening of Fungi with Molluscicidal Activity

Using a sterile inoculating loop, a loopful of isolate from stock as prepared in section 2.2 was inoculated on PDA and left in the incubator for seven days at 30°C. After seven days, about 10 mL of sterile water was poured on the agar, and the biomass was scraped and mixed with water. This suspension was transferred into a sterile universal bottle and vortexed and labeled as stock culture.

For screening purposes, 5 % (v/v) of stock culture was added to the PDA broth and left in a rotary shaker (Sartorius CERTOMAT® BS-1) at 150 rpm and 30°C for seven days. Next, the supernatant was separated from the cells by using filtration. The filtrates were prepared into two parts, where one was non-autoclaved while the other part was treated by autoclave for 15 min at 120°C. Both filtrates of 100% concentration in volume of 500 mL were poured into each plastic container with a 15 cm diameter and 11.5 cm height. Around ten snails of 2.93 ± 0.36 cm (length) and 6.40 ± 1.89 g (weight) taken from the growth tank were put into each container, with three replicates for each isolate used. A dead snail was removed, and the mortality of the snails was recorded. Only fungi showing molluscicidal activity were used for further identification.

2.4 Identification of Fungi

Identification was done through DNA sequencing [16]. The identification of isolated microorganisms was outsourced to Macrogen Inc., Korea, and MyTACG, Malaysia. Fungus mycelium was transferred into a 1.5 mL microcentrifuge tube containing 50 µL autoclaved-ultrapure water. This suspension was mixed vigorously using a vortex for 5 min and incubated at 95°C for 10 min. Next, the microcentrifuge tube was frozen at -20°C for 10 min. For PCR amplification, the cell suspension was centrifuged at 11,000 rpm for 5 min and 5 to 10 µL of the supernatant was used as a DNA template. The ITS-4 and ITS-5 primer was used for the amplification of the fungal ITS region. Each PCR reaction contained 1 x EconoTaq PLUS master mix (1.5 mM $MgCl_2$, 200 µM dNTPs, and 0.1 units/µL EconoTaq DNA polymerase) (Lucigen, USA) and 0.5 µM of each primer. The PCR conditions were 94°C for 10 min, 30 cycles of 94 °C for 20 s, 53°C for 20 s, 72°C for 1 min and lastly 72°C for 7 min. The expected size of the PCR product is 600 – 700 bp. The PCR product was purified using the TIANquick Mini purification kit (TIANGEN, China) according to the protocol provided by the manufacturer. DNA sequencing was done by Genomics BioSci and Tech, Ltd. (Taiwan) using ABI 3730/3730xl DNA analyzer (Life Technologies, USA).

3. RESULTS AND DISCUSSION

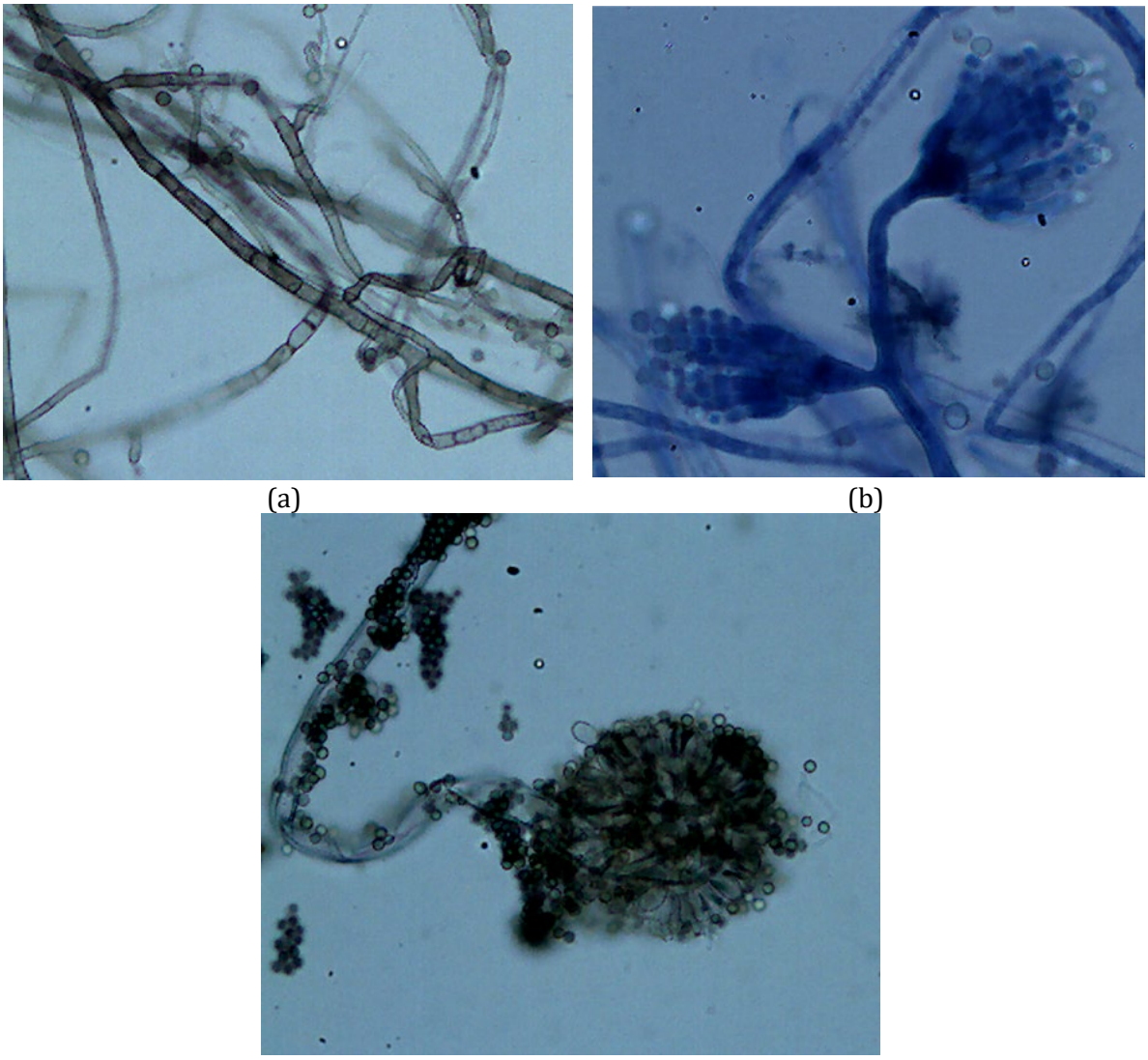
3.1 Morphological Characteristics of Isolated Fungi

There were nine types of fungi isolated from the dead snail collected from waterways in Kuala Perlis (KP). Most of the isolates grew on the edge of the agar surface due to a larger amount of resources available for their growth. Subculturing was done several times to isolate a single strain, prolong their life and also to expand or increase the cell population [16]. A mixed population of fungi was observed growing on agar with distinctive morphology.

Morphological characteristics of each isolate are expressed in Table 1. Based on the result, the types of asexual reproduction can be divided into three, which is sporangiospore, conidiospore and arthrospore. KP1 – KP5 are grouped into fungi with sporangiospore, KP6 – KP8 are fungi with conidiospore, while KP9 is a fungus with arthrospore. Figure 1 shows the image of fungal structure observed under a light microscope, including aseptate hyphae, a biseriate conidial head and a uniseriate conidial head. Isolate KP6 has a uniseriate conidial head with a long conidiophore and a round vesicle, whereas KP7 and KP8 have a biseriate conidial head and a short conidiophore.

Hyphae are a basic body unit of fungi in the form of a tube-like structure that grows, absorbs nutrients and forms reproductive structures [17]. Hyphae are divided by cross-walled septa, dividing the hyphae into individual, cell-like compartments. Some fungi consist of aseptate hyphae (coenocytic hyphae), which lack these cross-walls (septa), where their hypha forms a long, continuous tube of cytoplasm. Different groups of fungi are associated with different kinds of asexual reproduction. Some fungi have a sporangiophore, a fungal hyphae as a stalk that

supports a sporangium, a sac-like structure producing and holding spores, while others have a conidiophore, which bears asexual spores called conidia (conidiospores).



(a) (b)

(c)

Figure 1: Morphological characteristics of isolated fungi. (a) Aseptate hyphae (b) Biseriate conidial head, (c) Uniseriate conidial head.

Table 1: Morphological characteristics of isolated fungi.

				Reproductive characteristic					
Label	**Appearance on PDA**	**Sclerotia**	**Hyphae**	**Sporangiophore**	**Sporangia**	**Spores**	**Conidiophore**	**Vesicle**	**Type of Asexual reproduction**
KP1	Greenish Black	None	Aseptate		Round sporangia				sporangiospore
KP2	Brownish Black	None	Septate		Round sporangia				sporangiospore
KP3	Coffee Black	None	Aseptate	Long , unbranched	Round sporangia				sporangiospore
KP4	Grey	None	Aseptate	Long, unbranched	Round sporangia				sporangiospore
KP5	Yellow	None	Aseptate		Oval sporangia				sporangiospore
KP6	Green	√	Aseptate			Uniseriate	Long, colourless	Round	Conidiospore
KP7	Brown	None	Aseptate			Biseriate	Short, Colourless	Round	Conidiospore
KP8	Yellowish Orange	None	Aseptate			Biseriate	Short, Unbranched	Oval	conidiospore
KP9	White	None	Aseptate		Round sporangia				arthrospores

3.2 Screening of Selective Pathogenic Fungi

Molluscicidal activity refers to the ability of a substance to kill mollusks such as snails and slugs. This experiment evaluated the molluscicidal activity of various isolated fungi against GAS. As shown in Table 2, the mortality of GAS increased with the duration of exposure. Snails exposed to non-autoclaved fungal filtrates of fungi KP3, KP8 and KP9 show increasing mortality over 24, 48 and 72 hours, while the control group, KP2 and KP4, shows no mortality. KP3's non-autoclaved filtrate caused 23%, 37%, and 47% mortality, significantly higher than its autoclaved counterpart, which only caused 10%, 17%, and 27% mortality. Likewise, the non-autoclaved KP8 filtrate resulted in higher mortality (27%, 33%, and 50%) than the autoclaved filtrate (3%, 7%, and 20%). KP9 shows 13%, 20% and 27% mortality over time for the non-autoclaved filtrates, with no mortality observed in the autoclaved counterpart. Several fungi produce toxins such as aflatoxin and alkaloids, which act as either poison or hallucinogen and produce several kinds of toxins, including phalloidin and amanitin [16]. Some fungi species were reported to produce a toxin known as ochratoxin A and other secondary metabolites involved in pathogenicity [18,19].

Table 2: Mortality percentage of snails after 24, 48, and 72 h exposed to fungi filtrates. For each concentration, average based on three replicates ±SE, n = 30.

	Mortality percentage, %					
	24hour		**48hour**		**72hour**	
Colony	**Non-autoclaved filtrates**	**Autoclaved filtrates**	**Non-autoclaved filtrates**	**Autoclaved filtrates**	**Non-autoclaved filtrates**	**Autoclaved filtrates**
Control	0	0	0	0	0	0
KP1	0	0	0	0	0	3 ± 1.925
KP2	0	0	0	0	0	0
KP3	23 ± 1.925	10 ± 0	37 ± 1.925	17 ± 1.925	47 ± 1.925	27 ± 1.925
KP4	0	0	0	0	0	0
KP5	0	0	0	0	3 ± 1.925	3 ± 1.925
KP6	3 ± 1.925	0	7 ± 1.925	0	20 ± 3.33	0
KP7	0	0	3 ± 1.925	0	10 ± 3.33	3 ± 1.925
KP 8	27 ± 1.925	3 ± 1.925	33 ± 1.925	7 ± 1.925	50 ± 3.33	20 ± 3.33
KP9	13 ± 5.09	0	20 ± 3.33	0	27 1.925	0

3.3 Identification of Selected Fungi

Only isolates that show nearly 50% mortality were selected for further identification process. Results are as shown in Table 3. The DNA sequences for KP3 isolate are 99 % identical to *Aspergillus awamori,* while the DNA sequences of KP8 identify these isolates at the genomic level as *Talaromyces stipitatus* (also called *Penicillium stipitatum*). The morphology of the isolate is similar to that described by Stolk and Samson [20]. Several studies reported that *T. stipitatus* has been isolated from various environments such as mangrove roots, oceans and rice paddies, where water is the medium distributing this fungus [21-22].

Table 3: Blast report for KP3 and KP8 as referred to the database.

Label	Description	Accession	Identities, %
KP3	Aspergillus awamori strain K-03 18S ribosomal RNA gene, partial sequence	KF922319.1	99
KP8	Talaromyces stipitatus strain CRRI-59 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.	JX122729.1	99

4. CONCLUSION

For years, golden apple snail (GAS) infestations have threatened food security by damaging paddy seedlings and reducing agricultural yield. Even though chemical pesticides can control these invasions, they pose negative risks to both the environment and human health. This study intended to isolate pathogenic fungi from the flesh of dead snails to acquire a selective fungus capable of killing GAS without jeopardizing the surrounding ecosystem. From a total of nine fungal isolates, KP3 and KP8 revealed significant molluscicidal activity against GAS. Subsequent identification confirmed KP3 as *Aspergillus awamori* and KP8 as *Talaromyces stipitatus*.

ACKNOWLEDGEMENTS

The corresponding author is thankful to the Faculty of Chemical Engineering & Technology and the Faculty of Mechanical Engineering & Technology, Universiti Malaysia Perlis, for the support in providing the chemical and research facilities.

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Conflict of interest statement: The authors declare no conflict of interest.

Author contributions statement: Conceptualization, N. Zainalabidin & N.H.M. Salleh; Methodology N. Zainalabidin; Validation, N.H.M. Salleh; Writing & Editing, N. Zainalabidin; Supervision, N.H.M. Salleh, Funding Acquisition, N.H.M. Salleh