

OP-073 [Bee Biology]

Optimization of In-Vitro Rearing of Indo-Malaya Stingless Bee (*Heterotrigona itama*) Queen: A Strategy to Improve the Meliponiculture Management and Conservation of Insect Pollinator in Tropical Landscapes

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Malaysia is home to diverse species of Indo-Malaya stingless bees (Hymenoptera: Apidae: Meliponini), which consist of ~33 species. Stingless bees are known to be important pollinators in tropical rainforests and good candidates for providing pollination services in agricultural ecosystems. Due to the rapid advance of Malaysian meliponiculture in using the stingless bee as an agricultural pollinators, as well as the commercialisation of stingless bee products (pollen, honey, and propolis), the demand for colonies has considerably increased. Thus, an effort to multiply the number of stingless bee products rapidly must be developed in order to fulfill the public requirements in a short period of time. Current practice used by the stingless beekeepers is usually by cutting down the trees in the rainforest areas to obtain the bee colony brood and the queen bee. This concern has led to an alternative technique to mass-produce the queen on a large scale, particularly to our native species, *Heterotrigona itama*. To date, our research group has successfully developed an eco-friendly technique for rapid multiplication of the stingless bee colonies using in-vitro rearing procedure. The influence of the beneficial fungus *Monascus ruber* in promoting the survival of *H. itama* larvae was verified as 90% of larval survivorship was obtained when treated with fresh larval food compared with freeze-thawing sterilised larval food. Further experiments revealed that 150 µl and 120 µl of larval food have successfully yielded 78 % and 40 % of queen emergence, respectively. The in-vitro queens' abdominal length was significantly longer (4.24 ± 0.01 mm) than the workers' abdominal length (1.55 ± 0.009 mm). The validation of in-vitro queens is confirmed by the presence of huge ovariole numbers and spermatheca compared to workers that built with small ovaries and no spermatheca appearance. Outcomes obtained from this innovation enable the production of the colonies in a more sustainable and eco-friendly way, allowing for their use both ecologically and economically, and contribute to conservation efforts in Indo-Malaya stingless bees.

Optimization of In-Vitro Rearing of Indo-Malaya Stingless Bee (*Heterotrigona itama*) Queen: A Strategy to Improve the Meliponiculture Management and Conservation of Insect Pollinators in Tropical Landscapes



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Introduction

- Stingless beekeeping (**meliponiculture**) is one of the significant areas in the agricultural sector of Malaysia.
- The inclusion of honey as one of the Agro-resources and services in 12th Malaysia Plan, RMK12 (2021-2025) demonstrates the importance of beekeeping and its related activities in Malaysia's national agenda.
- It is an **emerging new source** of income for many Malaysian beekeepers & is common in Malaysia.
- In 2020, the total stingless bee honey production in Malaysia was recorded at 134,244 kg and generated total sales of RM19.3 million.
- High demand for stingless bee products (**honey, propolis, pollen**).
- Effective agricultural **pollination** – increase crop yield & quality.



Current Issues of Meliponiculture in Malaysia

- Demand for stingless bee colonies increased rapidly - **beyond the supplying capacity of beekeepers.**
- Current practice typically involves cutting down trees in rainforest areas to obtain the bee colony brood and the queen bee.
- The log hunting activity should be stopped immediately, due to the habitat loss of the rainforest ecosystem.
- Major bottleneck of meliponiculture in Malaysia:
 - **The rearing of the queen**
 - Low honey production
 - Quality inconsistency
 - High price
- Thus, an effort to multiply the number of stingless bee products must be developed in order to fulfil the public requirements in a short period of time.





The Role of Stingless Bee Queen

- Plays a vital role in the hive because she is the only female with fully developed ovaries.
- Three primary purposes:
 - controls the population of the hive
 - laying fertilised female eggs or unfertilised male ones
 - to produce chemical scents that help regulate the unity of the colony



Objective of the Study:

To develop an eco-friendly technique for rapid multiplication of the stingless bee colonies using an in-vitro rearing procedure.

General Methodology



Phase 1

- Effect of larval food amount on in-vitro rearing of stingless bee queen, *Heterotrigona itama*

Phase 2

- Effect of symbiotic fungus on survival of stingless bee queen, *H. itama* larvae

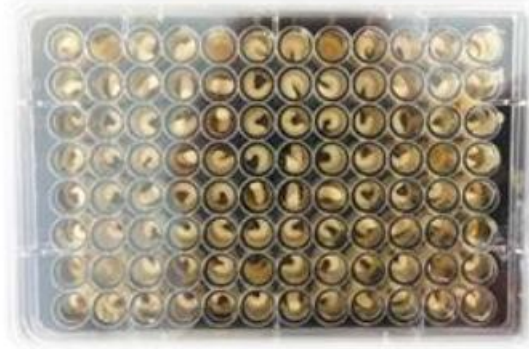
Phase 3

- Incubation period & life cycle of in-vitro stingless bee queen, *H. itama*

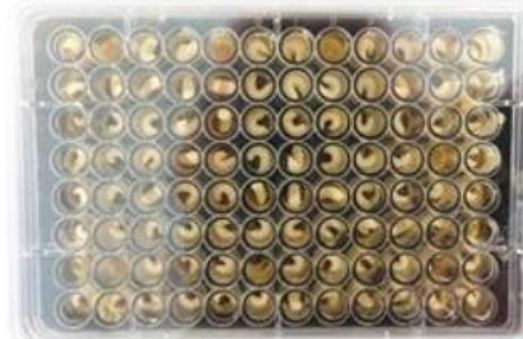
Phase 4

- Fertility and fecundity of in-vitro stingless bee queen, *H. itama* in field trials.

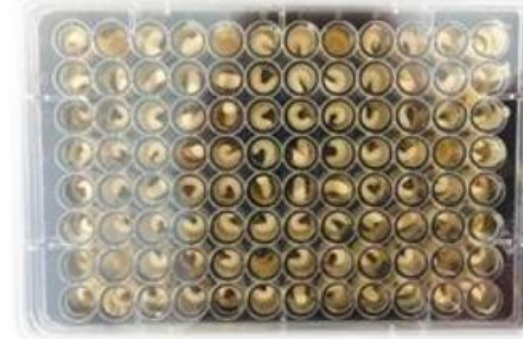
Experimental Design



Treatment A: 100µl of
larval food (control)



Treatment B: 120µl of
larval food



Treatment C: 150µl of
larval food

Total replicate for each treatment: 50 cells containing 50 eggs
Total number of samples: 150 eggs

Relative Humidity (RH):

100% -First 7 days

75% -The rest of larval
development

29°C -Temperature



Prior experiment, the there different amount were selected
after we measured larval food amount on several new brood
cells ($n = 20$) of *H. itama* workers ($25.4 \pm 1.22\mu\text{l}$)

*Parameters= Caste emergence (Kruskal-Wallis Test &
Survival growth pattern for each individual-Kaplan-
Meier)*

Experimental Design



Treatment A:
Autoclaved larval food
(control)



Treatment B:
Fresh larval food



Treatment C:
Autoclaved + supplemented with
symbiotic fungus

X



Total replicate for each treatment: 35 cells containing 35 eggs
Total number of samples: 105 eggs

100% -First 7 days
75% -The rest of larval
development

29°C -Temperature



- Fungus was cultivated in a 30G Broth (pH 6.0, HCl)
- Six folds of serial dilution with 0.02% of Tween 80 in dH₂O were prepared
- The organic phase of each folds were analysed using Chromatography and Mass Spectrometry meter
- 1µl of each inocula was tested into larval food (10 eggs)

Parameters= Larval survival (%) until Week 4

Isolation and Identification of Symbiotic Bee Fungus

Collection of Sample

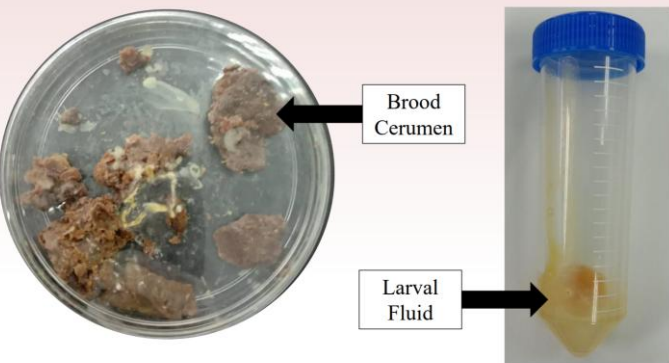
Preparation of selective media (ISP-2 agar medium)

Isolation of Fungus

Microscopic analysis

Molecular analysis

Samples



Preparation of ISP-2 Agar (30% glucose)

ISP-2 agar included:

- 30 g of glucose
- 3 g of malt extract
- 3 g of yeast extract
- 2 g of agar
- 100 mL of deionized water.
- Hydrochloric acid

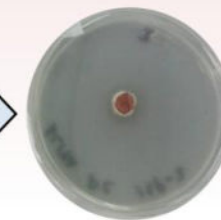


Preparation of Pure Culture

Fungus Colony Formed on ISP-2 Agar



Fungus Placed onto PDA agar

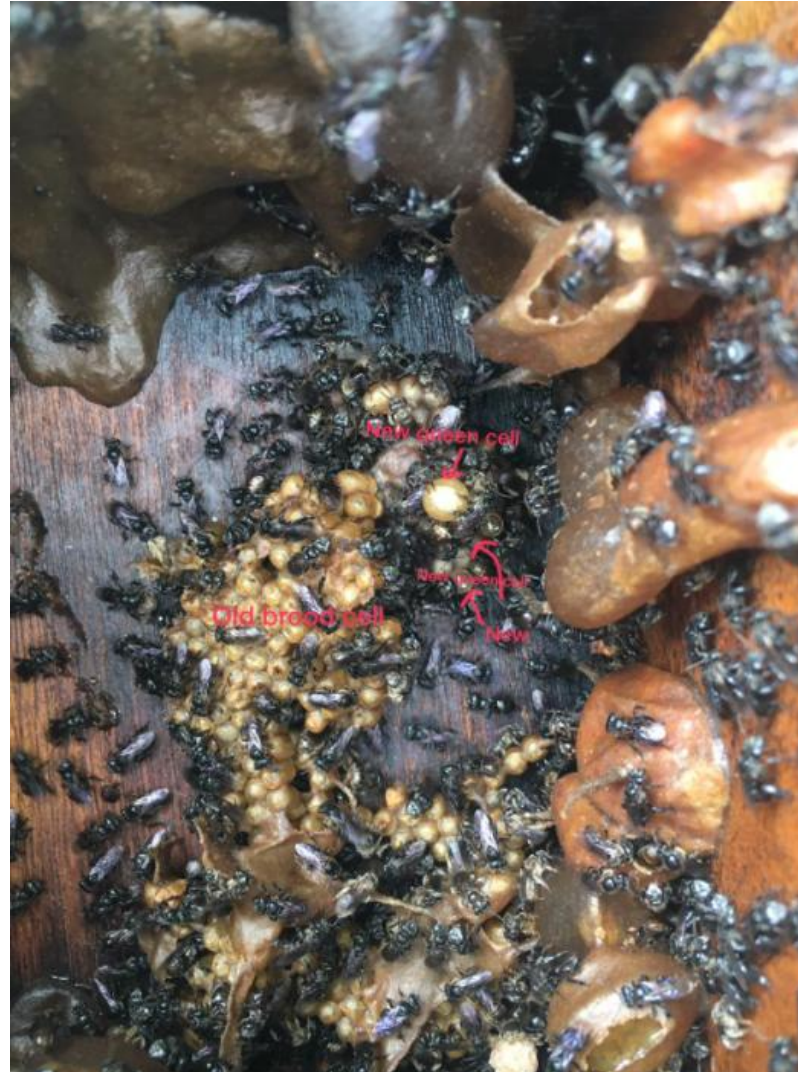


7-10 days

Pure Culture of Fungus Formed



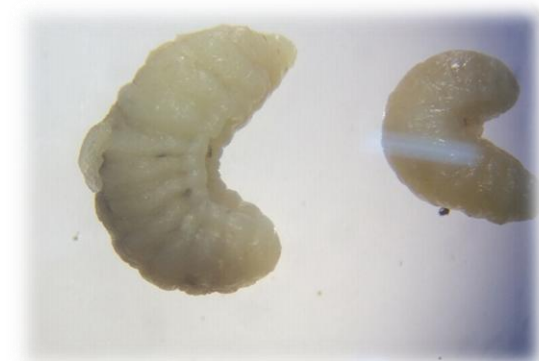
Introduction of in-vitro queen of *H. itama* to the new queen-less colonies in the field



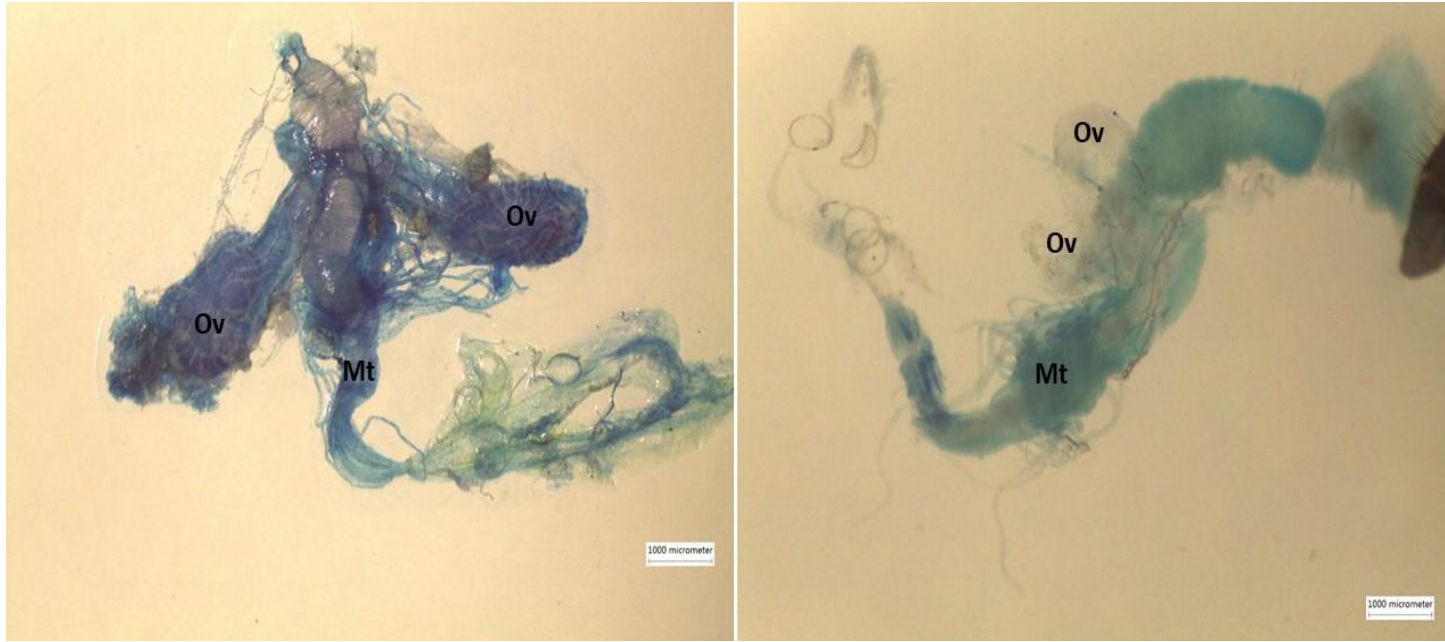
Results



- Larvae of *H. itama* treated with the highest amount of larval food (150 µL) led to 78% of the queen's emergence
- Whereas larvae treated with 120 µL and 100 µL of larval food resulted in 40% and 0% of queen emergence.



The percentage of worker and queen emergence respond to three different larval food amount from the preliminary study (N=150)



Microscopy images of gonada development in *H. itama* (A) Freshly dissected reproductive system of *in vitro* queen of *H. itama* with well-developed ovarioles structure, (B) Dissection figure of *H. itama* workers showed huge different of ovarioles size compared to *in vitro* queen (Ov, ovary).



FIGURE 4. *H. itama in vitro* queen reproductive system. The ovaries (Ov) built in with follicles connected to the lateral oviducts. The arrow exhibited spermatheca (Sp) connected to the similar oviducts. No spermatheca was observed in *H. itama* workers (Ov, ovary; Sp, spermatheca)



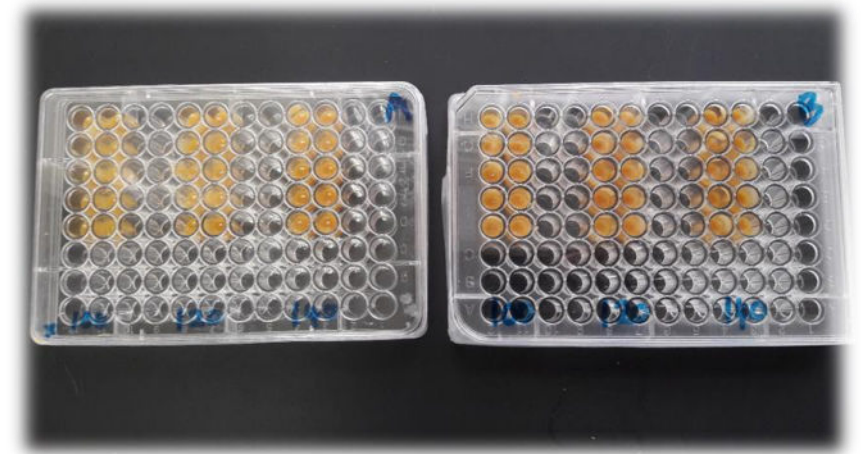
Figure 6. The anterior view of abdomen part of *H. itama in-vitro* queen. Scale attached in the figure

Results

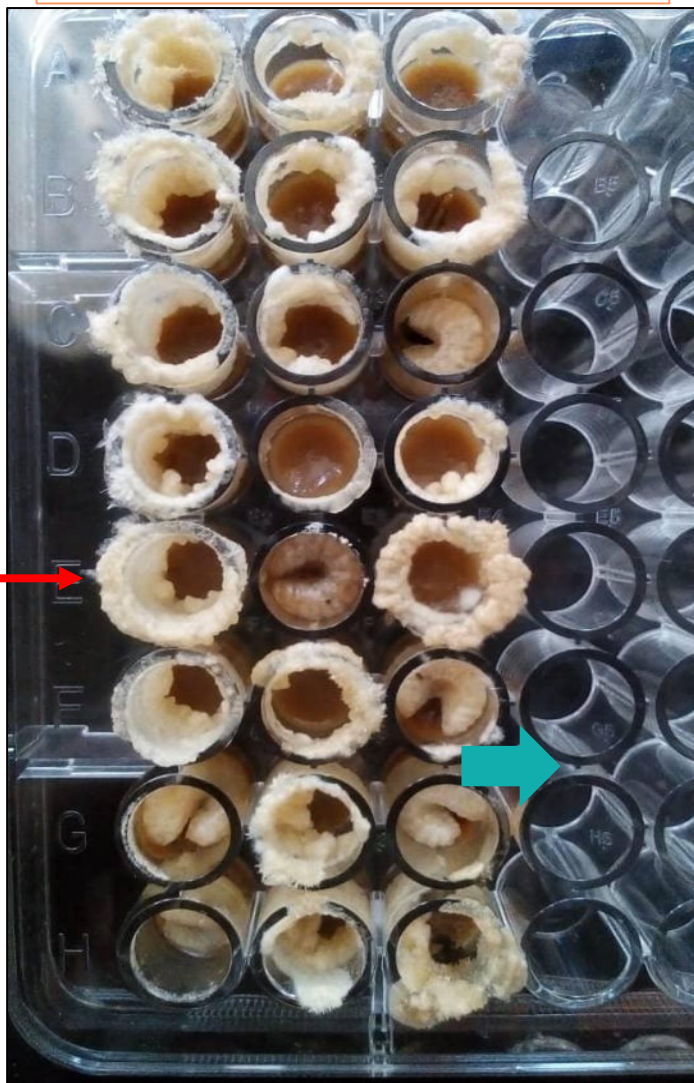
Table 1: Percentage of larval survival in response to three treatments (%) (n = 105).

	Treatment	Percentage (%)
Treatment 1	Microbes-free larval food	37.14
Treatment 2	Fresh larval food	85.71
Treatment 3	Microbes-free with fungus supplement	57.14

- Results showed that the survival of *H. itama* larvae depends on the presence of beneficial fungus with the highest survival rate (Treatment 2 = 85.71%).
- The absence of the beneficial fungus in the fluid food of *H. itama* showed the lowest survival rate (Treatment 1 = 37.14%).



TREATMENT B (with fungus)

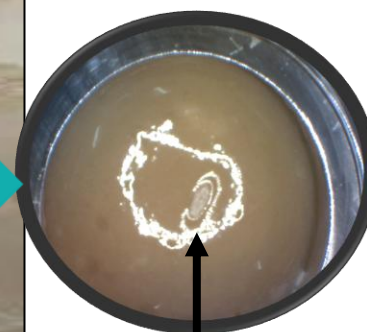
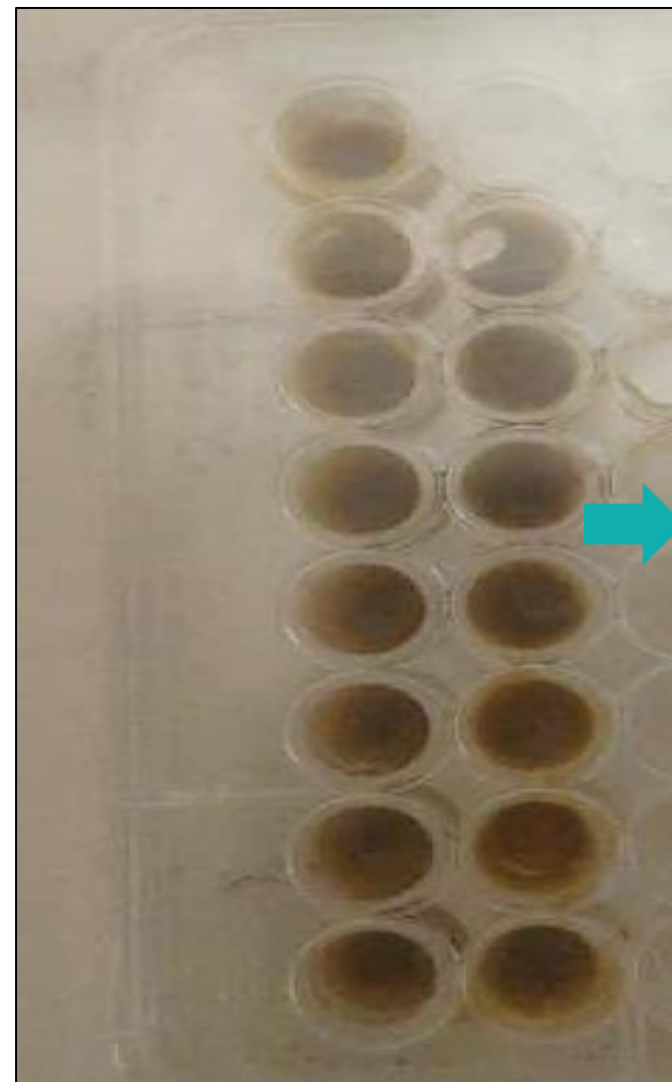


Fungus
appear
on the
border of
brood
cell

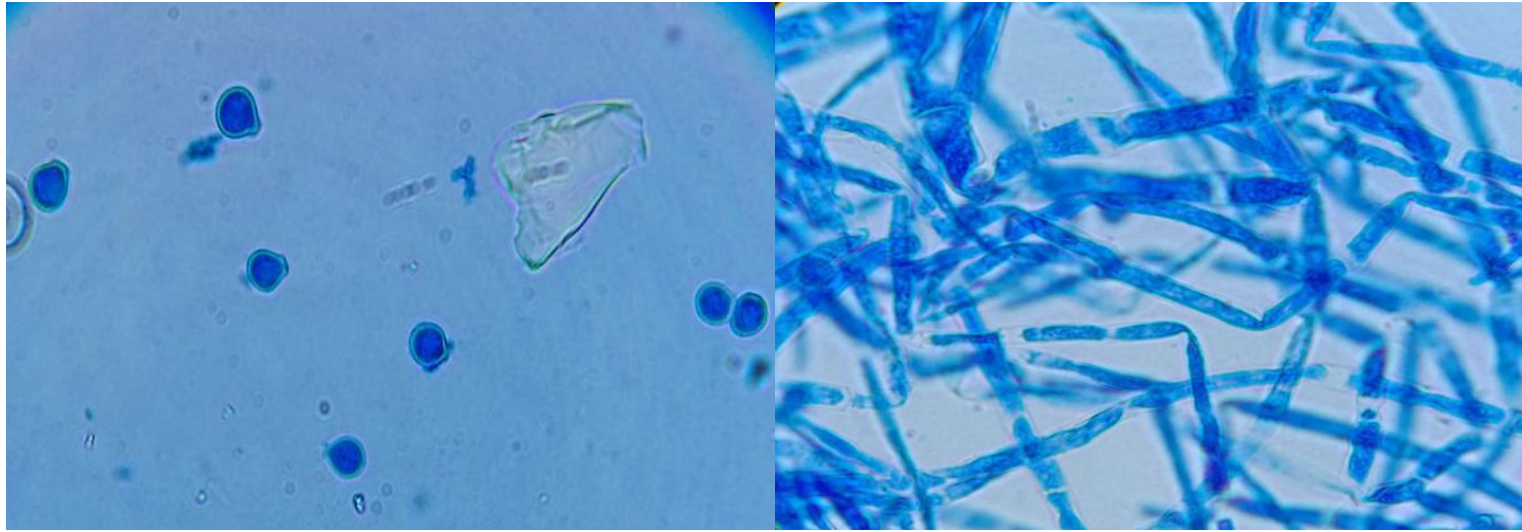


Larvae on
week 4

TREATMENT A (without fungus)



Not viable
egg



1000x magnification

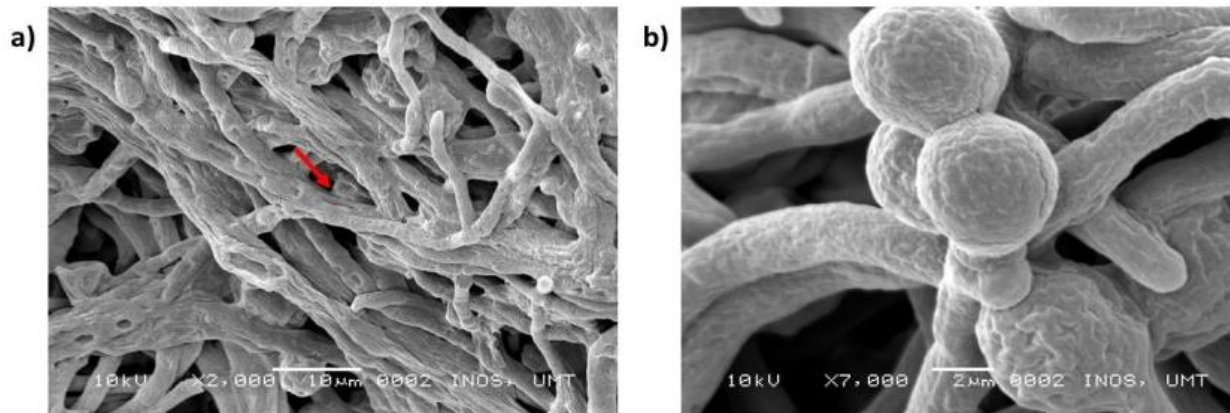
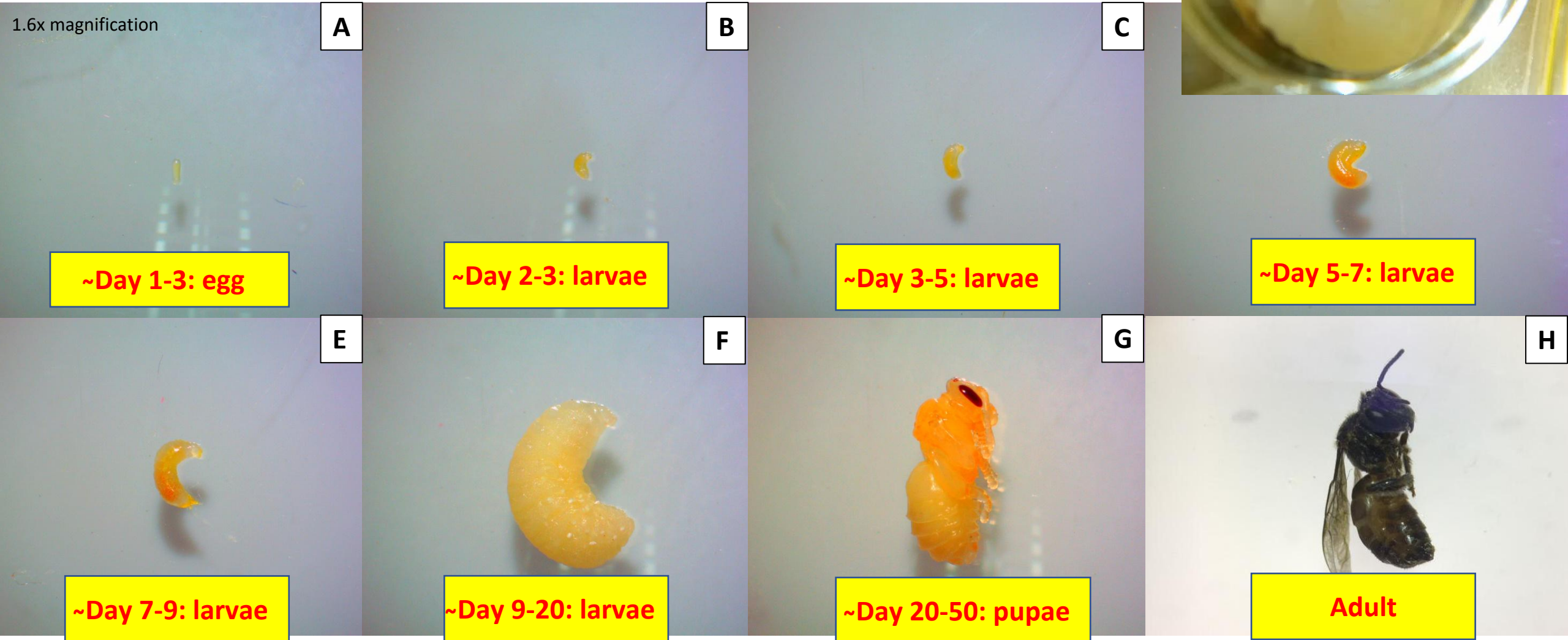


Figure 3: Scanning electron microscopy (SEM) analysis of *Panus lecomtei* at seven days of incubation period. (a) Arrow showed the dense of mature fibrous hyphal elements connecting between cells; (b) Budding formation of blastoconidia.

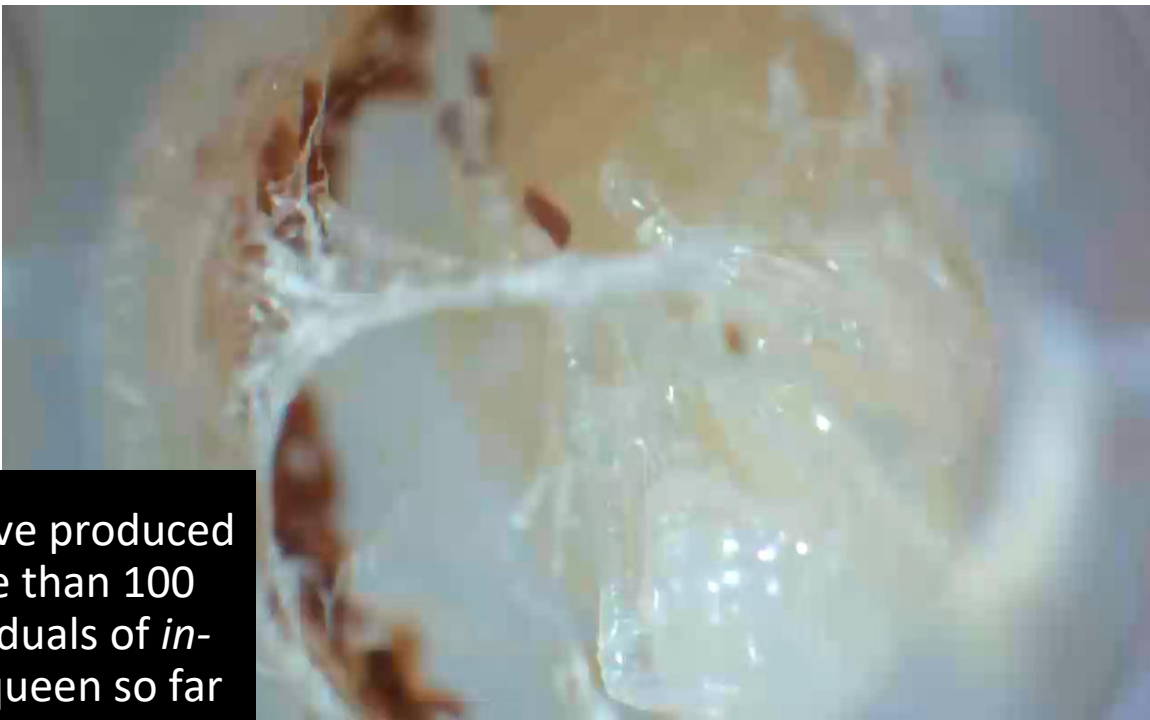
- Microscopy analysis showed that the fungus had various forms, including unconjugated asci, round-shaped ascospore and pseudohyphae.
- The molecular characterisation of the isolated fungi was performed using 23S rRNA gene sequencing using universal primers ITS1 and ITS4.
- DNA barcoding of three isolated fungi confirmed all isolated fungi matched *Panus lecomtei* (Basidiomycetes; Poriales; Polyporaceae) with 99.70% of similarity.

In-vitro Rearing and Growth Development of *H. itama*



A) ~1 to 2 day old egg; B) ~2 to 3 day old larvae; C) ~3 to 5 day old larvae; D) ~5 to 7 day old larvae; E) ~7-9 day old larvae; F) ~9 to 20 day old larvae; G) ~20-50 day old pupae; H) ~Adult of *Heterotrigona itama* (Queen)

We have produced
more than 100
individuals of *in-
vitro* queen so far





The success rate of queens' fertility has been tested in the field and results showed that more than 80% of newly developed colonies were successfully established from the new queens.

Conclusion

In-vitro Stingless Bee Queen



Practical, reliable
(conducted in a
controlled laboratory)
& repeatability

A new, simple and
sophisticated technique
to produce massive
stingless bee queens.

Effective method that could be
pre-adapted to tropical climate
of Malaysia.

Environmental friendly, safe, non-
destructive method, secure the
natural habitats, protect the
ecosystem balance and biodiversity.



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TERIMA KASIH

THANK YOU



Terokaan Seluas Lautan, Demi Kelestarian Sejagat | *Ocean of Discoveries for Global Sustainability*