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Effect of Wax-Coating on the Egg Hatchability of Golden Apple Snail, Pomacea Canaliculata

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ABSTRACT

Waxing is a common coating technique applied in postharvest handling that used to preserve the fruits and vegetables in order to maintain the quality of harvested fresh produce. This technique was then adopted in this study by spot spraying the egg masses of golden apple snail using different types of waxes. Hence, the objective of this study was to determine the effects of beeswax, carnauba wax and paraffin wax on the hatchability of golden apple snail eggs. The analysis of variance demonstrated that both single solution of waxes and admixed with synthetic molluscicides were significantly affect ($p<0.05$) the hatchability of golden apple snail eggs compared to control treatment. The results showed that increasing the wax concentrations were decreasing the hatchability of snail eggs, where the lower eggs hatching were found at 40.0mg/ml for beeswax in single solution and 60.0mg/ml of paraffin wax mixed with 0.36mg/ml niclosamide, respectively. The result was revealed the potential of fruit wax coating used as inhibitory agent to eggs-hatching of snail by forming an impermeable film covering the egg surface which altered the eggs development.

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INTRODUCTION

Golden apple snail (*Pomacea canaliculata*) is the amphibious freshwater mollusc that originated from Argentina (Wang *et al.,* 2012) has been considered as a threat to rice production in Malaysia. The presence of golden apple snail in irrigated rice cultivation area either intentionally or accidentally introduced have led to significants economic damage due to the loss of the plants (San Martin *et al.*, 2008). The damage level caused by the golden apple snail are depends on the rice crop stage, size and density of snail where one snail per square meter of medium-sized snail could reduced 19% of the number of rice tillers at 30 days after transplanting (Basilio, 1991; Naylor, 1996). In addition, several characteristics such as voracious appetite, high fecundity, able to aestivate in soil during dry season and have enormous reproductive capacity that owned by golden apple snail have led to the significant damage and its rapid multiplication (Du *et al.*, 2007). Joshi (2005) and Wang *et al.* (2012) have reported that golden apple snail able to lay eggs ranging from 20 to 500 eggs per cluster with 80% hatchability rate and reproduce three to four times per year. Thus, it was difficult to manage and control the golden apple snail invasion, once this pest infested the areas.

Despite various control strategies have been adopted to control the infestation of this pest in paddy field, but chemical methods using molluscicides are commonly and widely used by farmers. However, the application of molluscicide on juvenile and adults of golden apple snail is difficult as the snail could bury themselves in the soil (Dela Cruz *et al*., 2000; Dela Cruz and Joshi, 2001). Moreover, these molluscicides have lead various drawbacks and hazard to human health, ecology and environment. Concerns with this problem, it is imperative to explore the alternative control strategies that more effective and practical as well as environmental-friendly and socially acceptable.

Waxing is commonly known as a coating technique in postharvest handling of fresh produce which applied to the surfaces of fruits and vegetables in order to improve appreance and conserve the produces (Robert and Phillip, 1992). Studies in the past were demonstrated the application of wax on fruits surface have modified the internal atmospheres in fruits and prevent moisture loss during storage that prolong storage life of the fruits (Hoa *et al.*, 2002; Nadeem *et al.*, 2009). The barrier characteristics have hindered the ethylene (C_2H_4) and water vapour diffusion which attempt to retain the firmness of the fruits. It also form the diffusion barrier which is attributed to the inhibition of oxygen (O_2) and carbon

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dioxide (CO_2) exchange, thus reducing the respiration rate (Dou *et al.,* 1999). These findings have a theoretical value which suggests the hypothesis of the study where as the wax coating on golden apple snail eggs surface will affect its hatchability rate and embryonic development during incubation by modification of the internal atmospheres in eggs. According to Decuypere *et al.,* (2006), the availability of O_2 and CO_2 concentration in eggs is a fundamental importance for eggs development that may not only affect liveability of the embryo, but also affect hatchability as well as later development and functioning of the eggs. Additionally, Musman *et al.,* (2013) have stated that the factors such as low air supply to embryo, low temperature of incubation water and block water penetration into the egg were affect the hatching process of the golden apple snail eggs.

Golden apple snail was found to lay eggs in cluster at night by depositing them on the plant stems or trunks, concrete walls and stones above the water level to avoid desiccation or predation (Wu *et al.,* 2005; Horn *et al.,* 2008; Wang *et al.,* 2012). The bright pink colour of egg masses has made it highly visible and easy to find in the fields. Hence, it would lead to more easy and efficient control management of this invader by adopting the spot spraying on the egg masses. Since this approach is confined to egg masses, it has minimum environmental impact and give low cost of production to farmers. As it was much difficult to manage golden apple snail once they were hatching, a study is really required to control golden apple snail invasions as early and efficiently as possible. Thus, a study was initiated to discover the surface coating effect of lipid-based waxes that commonly used as edible coatings for fruits and vegetables on eggs. The purpose of this study was to determine the effects of carnauba wax, beeswax and paraffin wax on the hatching rate of golden apple snail eggs using spot spraying approach.

MATERIALS AND METHODS

2.1 Waxes and reagents:

The plant-based wax (carnauba wax), animal-based wax (beeswax) and petroleum derived wax (paraffin wax) were purchased from Sigma-Aldrich (M) Sdn. Bhd., Malaysia, while niclosamide (Bayluscide WP 70®) was purchased from commercial market. All other reagents used in the study were analytical grade obtained from ChemAR® Systerm, Malaysia.

2.2 Collection of golden apple snail eggs:

Fresh laid eggs of golden apple snail were randomly collected from the paddy field in Kota Belud, Sabah. Only egg masses that appeared freshly laid, as indicated by the most brightly pink colored eggs were collected. The egg masses were brought to the laboratory and subsequently placed individually in plastic containers that filled with 10.0 ml of distilled water.

2.3 Solvent selection by solubility determination:

The procedure for analysis the solubility of beeswax, carnauba wax and paraffin wax in selected diluents was performed according to Bernard *et al.,* (2011). Accurately weighed of wax was melted in a glass vial in water bath at temperature higher than its melting point. Then, required quantity of warm diluents such as chloroform, xylene, methanol and thinner was added to the melted wax and mixed well with a vortex for 20 seconds. The solutions were shaken mechanically for 12 hours in double-layer shaker incubator (SASTEC™, Model ST-250D, Malaysia) and, then allowed to equilibrate for a few hours, followed with centrifuged at 3000rpm for 5 minutes using K3 series refrigerated centrifuge (Centurion Scientific Ltd., UK). The supernatant of each solution was filtered through a 0.45µm syringe filter (Milipore, USA) prior analyzed spectrophotometrically using a Uv-vis spectrophotometer (SASTEC™, Model UV-8000, Malaysia) with wavelength range between 190 to 500nm for maximum absorbance (λ_{max}) determination. The solubility value of each wax in each solvent was calculated based on subtraction absorbance value of sample before and after the centrifugation. The analyses were performed in triplicate and the mean results are reported.

2.4 Exposure of treatment on golden apple snail eggs:

Wax coating of golden apple snail eggs was carried out and slightly modified from Wu *et al.,* (2005) procedure. The egg masses of golden apple snail were exposed to beeswax, carnauba wax and paraffin wax at five different concentrations ranging from 20.0 mg/ml to 100.0 mg/ml for a single solution and admixed with synthetic molluscicides (Bayluscide WP 70®) at a range from 0.36 mg/ml to 20.0 mg/ml. The each egg mass in each experimental set was sprayed with 5.0 ml of tested solution using a spray bottle. The treated egg masses were placed perpendicular against the wall in plastic container. The hatchability of eggs was observed for 20 days after treatment and data was taken at day 1,3,7,10,14 and 20. Golden apple snail eggs usually take 14 days for completion of hatching, so non-hatched eggs in each egg mass were separated in a 10% of sodium hydroxide (NaOH) solution and counted at day 20.

2.5 Microscopic analysis:

Microscopic examination was conducted to visualize the microstructure of the egg surface of golden apple snail, and then ascertain the bonding of a wax coating to the shell surface and the depth of penetration, if any. A visual assessment was examined using a trinocular polarized light microscope (Olympus model BX51, Olympus Corporation, Tokyo, Japan), equipped with 10x magnification and connected to a video camera.

2.6 Statistical analysis:

The efficiency of the fruit wax coating solution against the golden apple snail eggs were determined

by one-way analysis of variance (ANOVA) test followed by regression analysis. The α-level of significant differences was set at $p \leq 0.05$. All data were analyzed using the statistical software package of Minitab® Release 14.1 (Minitab, Inc.).

RESULT AND DISCUSSIONS

3.1 Solubility of waxes in solvents:

The maximum absorbance (λ_{max}) of wavelength for beeswax, carnauba wax and paraffin wax in each solvent are shown in Table 1. From the spectrophotometric scan, it indicated the wax that dilute in chloroform having the lowest λ_{max} of wavelength which was found at 248 nm, 339 nm and 230 nm, respectively. The highest λ_{max} was found in beeswax (449 nm) and paraffin (452 nm) diluted with methanol, where as carnauba was 468 nm when diluted in thinner. This maximum absorbance value was selected and used in estimation of wax solubility when the suitable diluents for each wax were found.

Table 1: Maximum absorbance (λ_{max}) of wavelength.

	Solvent (nm)			
Wax	Chloroform	Thinner	Xylene	Methanol
Beeswax	248	339	310	449
Carnauba	339	468	339	432
Paraffin	230	432	305	452

The solubility of three different waxes, beeswax, carnauba and paraffin wax in a number of solvents including methanol, thinner, xylene and chloroform were determined through visual observation (Figure 1 and Figure 2) and quantitative analysis using spectrophotometer (Figure 3). From the visual observation, all the three waxes were showed good solubility profile in chloroform (Figure 1). There is no precipitation of dissolved waxes observed when diluted with chloroform. However, the Figure 2 shows that only paraffin wax was slightly soluble in xylene and all the three waxes were insoluble in methanol and thinner. This behaviour is clearly observed that the waxes tends to agglomerate and starts to solidify in diluents after centrifugation. These results were support with the spectrophotometric analysis as shown in Figure 3, which the results obtained were significantly different ($p<0.05$) for all solvents employed and found that chloroform had the lowest absorbance difference before and after centrifugation.

This observed difference in the solubility of waxes can be explained on the basis of their carbon number distributions and the nature of the solvents (Srivastava *et al.*, 1997). The greater the relative concentration of the high-melting n-alkanes in the wax, the poorer is the solubility. Besides, the solubility of waxes in organic solvents is depends on a better or weaker interaction between the solute and the solvent molecules. In other words, it can be explained on the basis there is an attraction between the molecules in the solid and the solvent molecules when this liquid comes into contact with the wax surface. This attraction is slightly stronger than the attraction existing in the pure substances and so the wax dissolves into the solvent (Hargreaves, 2003). This work characterizes chloroform as a good and efficient solvent in dissolved waxes. Thus, this solvent was selected for further study.

3.2 Effect of waxes on eggs hatching rate of golden apple snail:

The efficacy of lipid-based waxes as egg-hatching inhibitor were evaluated at five different concentrations for a single solution and admixed with niclosamide against the golden apple snail eggs. After 20 days of observation, the result of eggs hatching were pooled across all the exposures time and non-hatched eggs in each egg mass were counted. The analysis of variance (ANOVA) among treatments demonstrated that both single solution of waxes and admixed with synthetic molluscicides were significantly affect the hatchability of golden apple snail eggs compared to control treatment (Table 2). In this study, exposure of golden apple snail eggs to single solution of beeswax, carnauba wax and paraffin wax caused a significant decrease in the hatchability of eggs which were $F=85.42$ ($p<0.05$), $F=24.63$ ($p<0.05$) and $F=35.88$ ($p<0.05$), respectively. In the second set of experiments, mixture solution of waxes and niclosamides were also affect significantly ($p<0.05$) in suppressing egg hatching of golden apple snail as shown in Table 2.

The relationship between the hatchability rate of snail eggs with the concentration of tested waxes and admixed with synthetic molluscicides are summarized in Figure 4 and Figure 5.

A visual inspection of the plot in both figures indicates a positive linear relationship between concentration of waxes and non-hatching rate of eggs, as the non-hatching rate of snails eggs increased with increasing the concentration of wax in single solution (Figure 4) and mixed with niclosamide (Figure 5).

Spraying the egg masses with single solution of wax determine the concentration of 20.0mg/ml for all waxes, and 40.0mg/ml for carnauba wax and paraffin wax including the untreated control group exhibit successful hatching of eggs. However, the hatchling rate of these wax treatments ranging from 9.97-23.85% were less than the 80% hatchability rate of the golden apple snail in the field as stated by Joshi (2005). Meanwhile, no egg hatching as reflected in

the 100% of non-hatched egg was observed at the concentration 40.0mg/ml and more for beeswax, and above 60.0mg/ml for carnauba wax and paraffin wax. It shows that the wax coating on golden apple snail eggs was significantly altered the normal development of the eggs. The developmental process of the embryos inside the egg is retarded as the waxes coating the eggs shell and blocks the eggs pores. The barrier characteristics have hindered the oxygen (O_2) and carbon dioxide (CO_2) from passing through the shell to the embryo thereby prevents the embryo from developing properly (Dou *et al.,* 1999; Demetillo *et* al., 2015). According to Decuypere *et al.,* (2006) and Horn *et al.,* (2008), the availability of O_2 and CO_2 concentration in eggs is a fundamental importance for eggs development that may not only affect liveability of the embryo, but also affect hatchability as well as later development and functioning of the eggs.

An effective concentration for each wax found in single solution experiment previously, which is 40.0mg/ml (beeswax) and 60.0mg/ml (carnauba wax and paraffin wax) were used in the second set of experiment by mixed with the niclosamide (0.36 mg/ml to 20.0mg/ml) at a ratio 1:1. Among the treatments in admixed solution as shown in Figure 5, a mixture of paraffin wax with niclosamide exhibited a significantly higher non-hatching rate of eggs with a maximum effect (100%) at the lowest concentration of niclosamide (0.36mg/ml). Moreover, treatment with beeswax at 10.0mg/ml of niclosamide and carnauba wax mixed with 15.0mg/ml of niclosamide caused 100% of non-hatched eggs, respectively. It was observed increase effectiveness of waxes on the hatchability rate of snail eggs with the increase in the concentration of the niclosamide, indicating dose-dependent activity. Thus, a mixture of 60.0mg/ml paraffin wax with 0.36mg/ml of niclosamide was found as the most effective solution in the inhibition of embryo development in eggs. Literature records have shown the treatment of golden apple snail eggs with niclosamide at 0.36mg/ml caused egg hatching inhibition of 13.7%, which could be classified as not effective in inhibiting egg hatching (Mohd Hafezan *et al.,* 2014). This is due to lack of penetration of niclosamide into the inner side of eggs as the egg surface was covered with solid colloid (Wang *et al.,* 2012). However, in this study it noticed that a mixture of niclosamide with wax have improved their performance and ability to control eggs hatching of golden apple snail effectively.

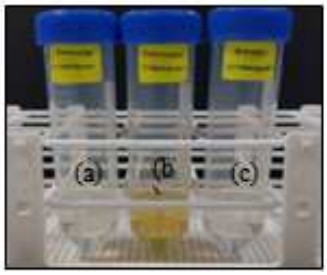

Fig. 1: Physical appearance of beeswax (a), carnauba wax (b) and paraffin wax (c) dissolved in chloroform after centrifugation process.

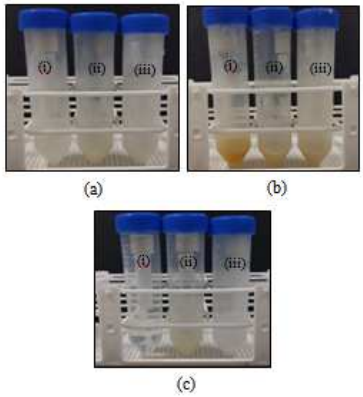

Fig. 2: Physical appearance of waxes; beeswax (a), carnauba (b) and paraffin was (c) dissolved in xylene (i), benzene (ii) and methanol (iii) after centrifugation process.

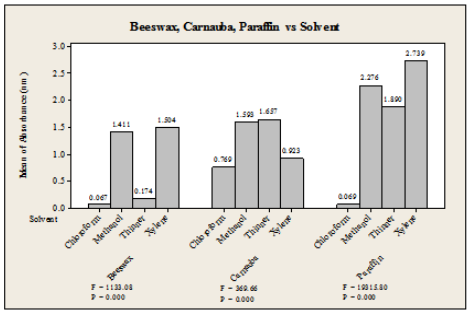

Fig. 3: The spectrophotometric analysis on the absorbance difference of wax solubility in solvent employed.

Table 2: Analysis of Variance (ANOVA) of the non-hatching eggs on the different treatments.

Treatment	Variation source	Sum of squares (SS)	Mean Square (MS)	F-value*	**Sig.
Single solution	Beeswax	20631.7	4126.3	85.42	0.000
	Carnauba wax	19717.0	3943.0	24.63	0.000
	Paraffin wax	19277.0	3855.0	35.88	0.000
Admixed solution	Beeswax	27503.0	5501.0	10.53	0.000
	Carnauba wax	16276.0	3255.0	27.58	0.000
	Paraffin wax	21006.9	4201.4	121.00	0.000

* Significant value: *df* = 5
**Significance at 5% level

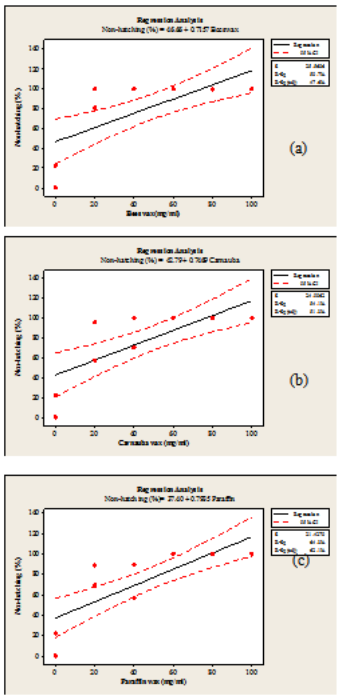

Fig. 4: Non-hatching rate (%) of golden apple snail eggs treated with different concentrations of beeswax (a), carnauba (b) and paraffin wax (c) in single solution.

3.3 Microscopic analysis:

The microstructure of the shell surface of golden apple snail eggs for untreated and treated with wax and niclosamide are presented in Figure 6. Coarse, dry and uneven whitish particles (show by arrow) were observed on the shell surface of untreated eggs (Figure 6a) which due to the egg surface was covered with solid colloid (Wang *et al.*, 2012).

After application of the wax, the coated eggs were characterized by the appearance of glossy film (show by arrow) covering the surface of the egg shells (Figure 6b). The image shows that the egg surface treated with wax coating was formed a smooth, thin and highly adherent additional layer on the shell surface which proves they had been act as a physical barrier for gases exchange into the eggs. Our observations are consistent with the reports of Meyer ans Spencer (1972) and Meljan *et* al., (2015) who claim that wax coating causes a blocking of shell pore canal on the egg surface, which results in

hindered of the diffusion of gases, retarded embryos development and inhibited the hatchability of the eggs. When the eggs treated with niclosamide, the droplets were not stay stable and not entirely covered the egg surfaces (show by arrow), leading to form poor coating film with poor gases and water vapour barrier (Figure 6c). Referring to all images in Figure 6, there was no penetration of the coating below the egg shell which the coating was only forms on the surface of tested eggs. It was due to the presence of solid colloid that serves as protector and covering the golden apple snail eggs surface.

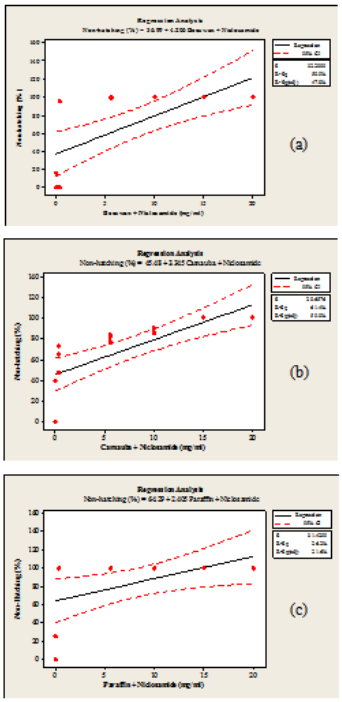

Fig. 5: Non-hatching rate (%) of golden apple snail eggs treated with a mixture of beeswax (a), carnauba (b) and paraffin wax (c) with different concentrations of niclosamide

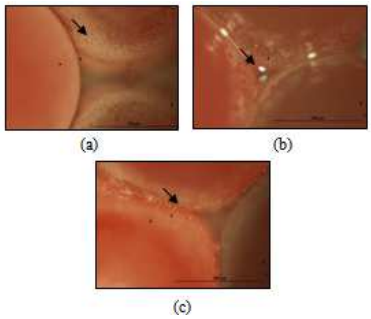

Fig. 6: Microscopic images showing external surface of the golden apple snail eggs for untreated eggs (a) and treated with wax (b) and niclsoamide (c).

Conclusions:

This study shows the most common waxes applied in fruit coating; beeswax, carnauba wax and paraffin wax gave significantly affect on the hatchability of golden apple snail eggs. The observations have suggested that the hatchability of snail eggs could be controlled in two ways through spot spraying approach either using a single solution of wax or admixed wax with niclosamide at recommended concentrations. In a single solution, it demonstrated that the beeswax at 40.0mg/ml was the most effective treatment in the inhibition of eggs development with 100% of non-hatched eggs, while a mixture of paraffin wax with 0.36mg/ml niclosamide also presented the similar maximum effects. Non-hatching rate of snail eggs were directly proportional to the increase of wax concentrations, which is attributed to the highest concentration gives the highest reduction of the eggs hatchability. The result was revealed the fruit wax coating can be potentially used as inhibitory agent to eggs-hatching of snail by forming an impermeable film covering the egg surface which could altered the eggs development. Nevertheless, a further study is needed

to determine the efficacy of this control technique assessed under field condition.

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