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# ANTIFUNGAL PROPERTIES OF WATER EXTRACT PROPOLIS COATING AGAINST ANTHRACNOSE (*Colletotrichum gloeosporioides*) DISEASE ON STRAWBERRY (*Fragaria anannassa*)

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## ABSTRACT

Anthracnose is a major pre and post-harvest disease. Due to the consumer concerns of the residual effect of the synthetic fungicide, biocontrol from a natural source is highly required by the consumer to reduce the problem. Propolis is a natural antifungal that can be used as a coating to control fruit quality and postharvest losses. This study evaluated the physical and chemical characteristics of strawberry (*Fragaria ananassa*) induced with *Colletotrichum gloeosporioides*, coated with water extract propolis (WEP) and stored at 5°C. The effect of coating was evaluated with different concentrations of water extract propolis (WEP) which were control, 10%, 12%, and 14%. The post-harvest parameters such as weight loss, total soluble solids (TSS), anthocyanin, disease severity index (DSI), and firmness were evaluated at two days interval for 12 days of storage. The highest percentage of inhibition (*in vitro*) was shown by the highest concentration of WEP (14%). Fruits coated with propolis showed reduced deterioration and infection diameter of fungus, as compared to the control (without coating) which already spoiled at day 4. Strawberry coated with 14% concentration of WEP also showed the highest firmness compared to other treatments. Thus, this coating can be used as an alternative method for bio-control of disease to reduce the post-harvest loss of fresh produce.

**Key words:** Propolis, stingless bee, antifungal, anthracnose, strawberry, post-harvest quality

## INTRODUCTION

Strawberry (*Fragaria ananassa*) is one of the perishable fruit, especially after harvest. The main causes of strawberry deterioration during storage are the development of rots that are caused by a range of fungi (Feliziani & Romanazzi, 2016). The most fungus that causes postharvest losses on strawberries is *Colletotrichum* species associated with strawberry anthracnose (Ellis & Erincik, 2008). The symptom randomly distributed gray to black spots caused by *Colletotrichum fragariae* or *Colletotrichum gloeosporioides* (Suzuki *et al*., 2010). *Colletotrichum gloeosporioides,* is one of the world's most important pathogens that cause anthracnose to most fruit (Phoulivong *et al*., 2010). Anthracnose is a major pre and post-harvest disease. However, fungicide schedule applications do not always result in efficient and the increasing of the fungi resistance to some active principles has been reported (Kososki *et al*., 2001). Rapid and constant cold storage added with coating may help in reducing the growth rate of microorganisms in strawberries (Ribeiro *et al*., 2007; Feliziani & Romanazzi, 2016). To date, there was a limited study on the biological control reported on reducing the anthracnose on strawberries especially from agriculture by-products such as propolis. Currently, there is an increasing interest in the use of organic compounds such as propolis as edible fruit coating because of its potential to inhibit fungal growth and extend the shelf life of fruits (Awawdeh *et al*., 2009). Propolis which was reported with excellent properties of anti-inflammatory, antibacterial, antiviral, immunomodulatory, antioxidant, and antiproliferative may act as the control for anthracnose (Viuda *et al*., 2008). There was almost no study reported on the stingless bee propolis as fruit coating. However, Ahmad *et al*. (2019) has reported on the great antimicrobial properties of

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propolis from stingless bee towards most of the food pathogen. Besides, a study was done by Zahid *et al*. (2013) emphasized the artepillin-C as the bioactive constituent form in propolis that related to the extremely potent antifungal properties of propolis. All of these studies indicate that stingless bee propolis has a high potential to be used as fruit coating too to reduce the fungal disease during storage. Thus, this study aims to study the potential of propolis from the stingless bee as an antifungal agent in reducing the anthracnose disease on the strawberry while maintaining the post-harvest quality during storage.

## MATERIALS AND METHODS

### Sample preparation

Raw propolis was collected from beehives located at Institut Perguruan Malaysia, 22200 Besut, Terengganu. Samples were cleaned by removing dead beed or any contamination and stored at -20°C for 20 min (Sun *et al*., 2015). Frozen propolis was grinded into a fine powder.

Strawberries with uniform size and color (75% red surface), free of physical damage and fungal were collected from Farm Agro Highland, Cameron Highland. Strawberries were washed using acetic acid (3%) and rinsed with distilled water and left air-dried at ambient temperature.

### Water extraction of propolis

A total of 400 g of propolis powder was homogenized with 1000 mL of water at 50°C (Thomas *et al*., 2016). The filtered extract was placed into a 50 mL Falcon tube and centrifuged at 10000 rpm for 10 min to obtain the supernatant. The supernatant was dried with a rotary evaporator (40°C). The paste was evaporated in a vacuum oven at a temperature of 60°C to obtain dry water extract propolis (Paviani *et al*., 2012) and diluted with sterilized water, to produce the concentration of 10%, 12%, and 14% of water extract propolis (WEP).

### Antimicrobial analysis (*in vitro*)

The antimicrobial analysis of water extract propolis (WEP) against *Colletotrichum* species was done using the poisoned plate technique (*in vitro*). Poison plate is a standard method that is normally used to determine the inhibitory effect of plant extract against fungal species such as *Colletotricum* sp. This method was done by placing the isolate of the fungus onto the agar mixed with extract and the inhibitory effect was observed after the incubation period (Das *et al*., 2010). In this study, the identified *Colletotrichum* sp. of strawberry was grown onto Potato Dextrose Agar (PDA). The culture was flooded with sterile distilled water and dislodged the spore using a glass rod. The spore suspensions obtained were passed through filtration of sterile cheesecloth to remove mycelial fragments (Lane *et al*., 2012). Spore suspension ($2 \times 10^6$ conidia/mL) was obtained by using a Haemocytometer (Sigma-Aldrich) counting. Different concentrations; 10% WEP (v/v), 12% WEP (v/v) and 14% WEP (v/v) was added into PDA and to solidify and tested against *Colletotrichum* sp. A 5 mm disc from seven days old of *Colletotrichum* cultures was plucked out and transferred to the middle of PDA and incubated at 28 ± 2°C for a week. The growth of the fungal colony was recorded. The absence of growth in the plate indicates the effectiveness of plant extract against the test. The two readings in the control and treatments was transformed into percent inhibition (%) of radial growth by using the following formula (Marinho *et al*., 2018):

Formula 1:

$$mycelium\ inhibition = \frac{(C - T)}{C} \times 100$$

Explanation:
C = colony diameter (mm) growth in the control.
T = colony diameter (mm) growth in the treatment.

### Application of propolis on strawberries (*in vivo*)

Strawberries were dipped for 2 min into water extract propolis (WEP) containing 10%, 12%, and 14%. All samples were inoculated by spraying prepared spore suspension of 3 x $10^6$ conidia/mL of *Colletotrichum gloeosporioides*. All inoculated and treated samples were kept at ambient temperature and analyzed at a 2-day interval for 12 days of storage for post-harvest quality.

### Post-harvest analysis

All samples were analyzed for post-harvest quality to determine the effect of coating (*in vivo*) from WEP on the fruit quality during storage. The post-harvest analysis was weight loss, total soluble solids, firmness, disease severity assessment, and anthocyanin. Weight loss of strawberry was done to see if the coating from WEP reduced the anthracnose occurrence then the respiration will be reduced and weight loss will be delayed. Weight loss was determined using an electronic balance (A & D Model HT-500). The result was expressed as a percentage of weight loss (%) according to the following formula (López-Castañeda *et al*., 2010):

Formula 2:

$$\%\ Weight\ loss = \frac{(initial\ weight - final\ weight)}{initial\ weight} \times 100$$

**Table 1.** Scale for disease severity assessment (Priyadarshanie & Vengadaramana, 2015)

| Disease scale | Description | Inference |
|---|---|---|
| 0 | No symptoms on fruits | No infection |
| 1 | 1–25% of the inoculated area covered with lesion | Mild infection |
| 2 | 26–50% of the inoculated area covered with lesion | Moderate infection |
| 3 | 51–75% of the sample are infected and softening | Severe infection |
| 4 | >76% and rotting | Very severe/ devastating |

The total soluble solids (Brix) were determined using a handled refractometer (Atago, MODEL REF 103) to determine the sweetness of the fruit. The fruit firmness was determined using TA*Plus* Texture Analyser (Stable Micro Systems) and the result was expressed as newton (N). Fruit firmness analysis is important to determine the freshness of the strawberries during storage with the disease. Disease severity assessment of the fruit was determined by observing the diameter lesion of anthracnose. The assessment was done based on the given scale (Table 1).

The overall disease severity index (DSI) was calculated as follows (Mak *et al*., 2004):

Formula 3:

$$DSI\ (\%) = \frac{\Sigma\ (number\ of\ symptomatic\ fruits\ x\ severity\ index)}{N \times Z} \times 100$$

N = Total of sample fruits.
Z = Highest rating scale.

The total anthocyanin content (TAC) was determined by the pH-differential method (Giusti & Wrolstad, 2001). Fruit (5 g) was grind with 10 mL methanol and centrifuged (5000 rpm, 10 min, 2°C). After adjustment of the sample with pH buffer (pH 1.0 with KCL and pH 4.5 with $CH_3COONa$), the extracted solution was then read using a spectrophotometer (Shimadzu UV-1800). The concentration of TAC was calculated based on the following formula (Damsa *et al*., 2016):

Formula 4:

$$A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$$

$$Monomeric\ anthocyanin = (A \times MW \times DF \times 100) / \mu / L$$

$$Pigment\ (mg/L)$$

A = Absorbance.
MW = Molecular weight (484.84 g/mol for cy-3-glu).
DF = Dilution factor.
ε = Molar extinction coefficient (34300 for cy-3-glu).
L = Path length.

**Statistical analysis**

The experimental design for this study is a complete randomized design (CRD). The data were subjected to analysis of variance (ANOVA) using IBM SPSS 23.

## RESULTS AND DISCUSSION

**Mycelium inhibition (%)**

The inhibition of *Colletotrichum gloesporioides* mycelium was increased with the increment of concentration of water extract propolis after a week incubation (Table 2 and Figure 1). The slower growth of tested fungi in the plate indicates the effectiveness of WEP against the fungi. The results are in agreement with an earlier study by Bussaman *et al*. (2012) who reported the increasing concentration of plant extract reduced the mycelial growth of *C. gloeosporioides*. A study by Kim *et al*. (2014) also showed the reduction of mycelial growth of *C. gloeosporioides* with *Streptomyces* sp. Results from this study also showed a similar percentage (%) of mycelial inhibition with the study by Kim *et al*. (2014). But, all of these previous studies use methanol extract while this current study used water extract. Water extract of the plant normally was reported with lower antifungal properties compared to methanol and other organic solvents (Jabeen and Javaid, 2008). The great antimicrobial properties of water extract propolis (WEP) as fruit coating in this study may be contributed by the high content of phenolic compounds in propolis (Ahmad *et al*., 2019). There are many studies reported on the positive

**Table 2.** Percentage of mycelium inhibition (%) using different concentration of water extract propolis concentration on PDA after 7 days of incubation

| Treatment | % Mycelium inhibition |
|---|---|
| 0% (Control) | 21.95 |
| 10% | 39.2 |
| 12% | 41.46 |
| 14% | 48.78 |

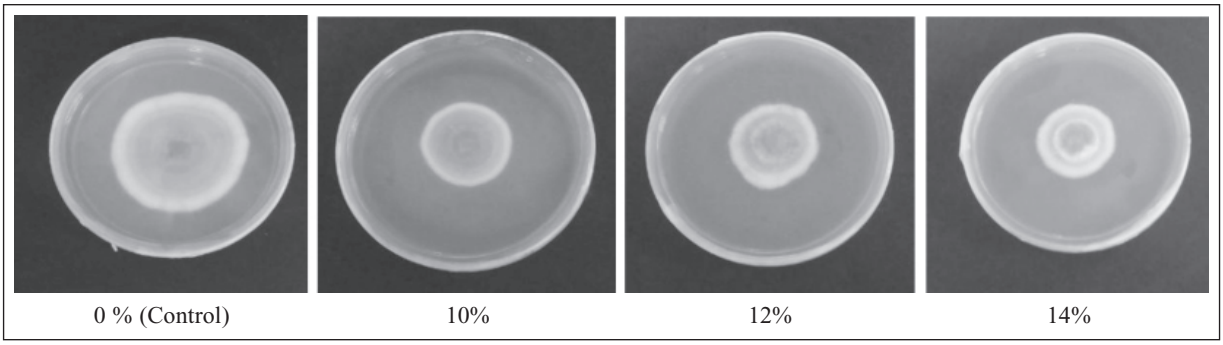


**Fig. 1.** The inhibition zone of *Colletotrichum gloesporioides* on poison plate with different concentration of WEP.

relationship between the antimicrobial properties and phenolic compounds (Alves *et al*., 2014; Ahmad *et al*., 2019) such as cinnamic acid, taxifolin, chlorogenic acid, and chicoric acid (Qadir *et al*., 2017). Besides, the presence of pinocembrin, galangi, and pinobankin in the flavonoid of propolis was also had antimicrobial properties (Park *et al*., 1998).

**Water loss**

Although the trend of weight loss of coated strawberry with WEP in Figure 2 showed a lower value there was no significant difference ($p>0.05$) between treatments. The physical appearance of non-coated fruit (Figure 7) showed severe infection of fungal but looks like it did not significantly affect the weight. This is maybe due to the propolis coating mixture which only consists of WEP and sterilized distilled water. The WEP is effective in reducing the anthracnose disease (Figure 6) but not sufficient to delay the water loss significantly. However, on day 12, the weight loss of fruit coated with 14% of WEP showed lower weight loss. This is due to the antifungal properties of WEP which delay the respiration rate of the fruit due to the infection by the fungus. Besides, the hydrophobic character of the propolis also resulted in a lower water reduction through transpiration and respiration (Barrera *et al*., 2015).

**Firmness**

Figure 3 showed the firmness of coated fruit (with WEP) was higher than the uncoated ($p<0.05$) during storage. Theoretically, firmness was correlated with respiration where carbohydrate was broken down by hydrolytic enzymes such as pectin methylesterase (PME) and polygalacturonase (PG) which results in a reduction of firmness during respiration (Chitarra *et al*., 2005). So, this hypothesis correlated with this study which showed the hydrophobic characteristic of propolis that can reduce the respiration and transpiration and subsequently reduce the loss of firmness. In addition to that, a study by Anjum *et al*. (2019) found that propolis rich with wax which makes contributes to the hydrophobic characteristic.

**Total soluble solids**

Theoretically, total soluble solids (TSS) of fruit increased with the advancement of ripening of fruits irrespective of maturity condition (Moneruzzaman *et al*., 2009). Any treatment that can delay the ripening during storage should result in lower TSS. However, in this study, there was no significant difference ($p>0.05$) reported for coated and non-coated strawberries (Figure 4) indicated that coating with WEP did not significantly delay the ripening of the fruit. The lower trend of TSS with the storage also showed that the ripening is not occurred as usual which may be affected by the infection of the disease.

**Anthocyanin**

The amount of anthocyanin (TAC) is important for the attractiveness and maturity assessments of strawberries. The result showed that TAC (Figure 5) was not affected by the application of WEP of the fruit ($p>0.05$). All treatments showed similar anthocyanin values during storage. The decreasing trend of anthocyanin may due to the fungistatic properties which secrete secondary metabolite which capable to degrade any phenolic compounds (Daayf & Lattanzio, 2008).

**Percentage of disease severity (DSI)**

Potential propolis as an antifungal agent could be potentially exploited in controlling the growth of phytopathogenic fungi during post- and pre-harvest (Meneses *et al*., 2009). There is a reduction in the severity of disease with increasing of WEP concentration (Figure 6). The disease severity of fruit was clearly shown in Figure 7 which indicates the negative result for *Colletotrichum gloesporioides* on all samples. All fruits did not show the symptom of *Colletotrichum gloesporioides* which distributed gray to black spots (Suzuki *et al*., 2010). However, there was another symptom that appears on the fruits (water-soaked with white,

**Weight loss**

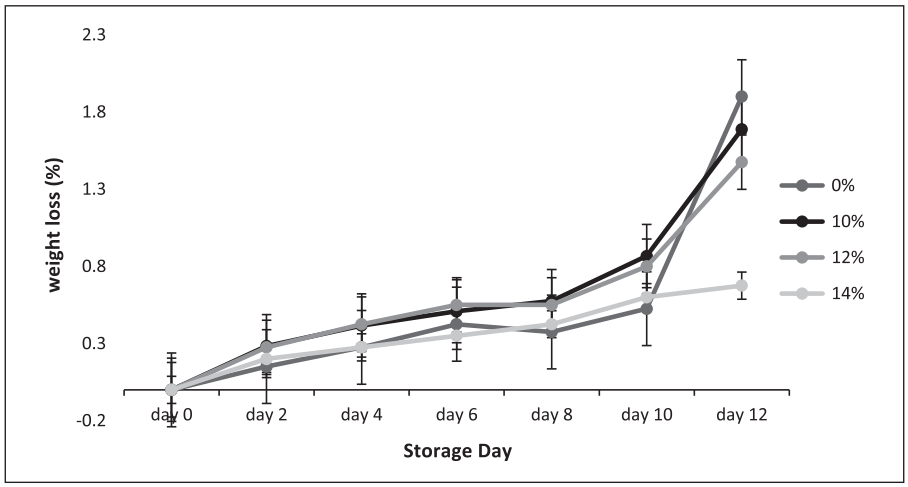


**Fig. 2.** Effect of the different water extract propolis concentration on the percentage of weight loss of strawberry.

**Firmness**

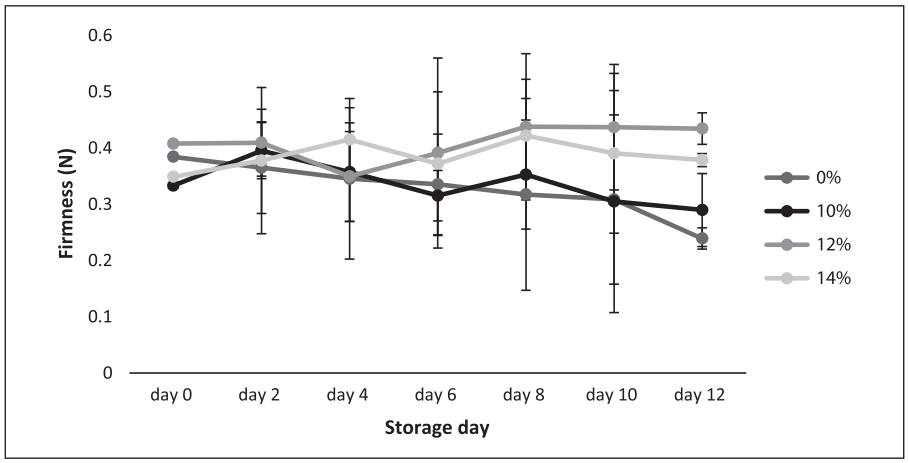


**Fig. 3.** Effect of the different water extract propolis as a coating on the firmness of strawberry.

**Total soluble solids (TSS)**

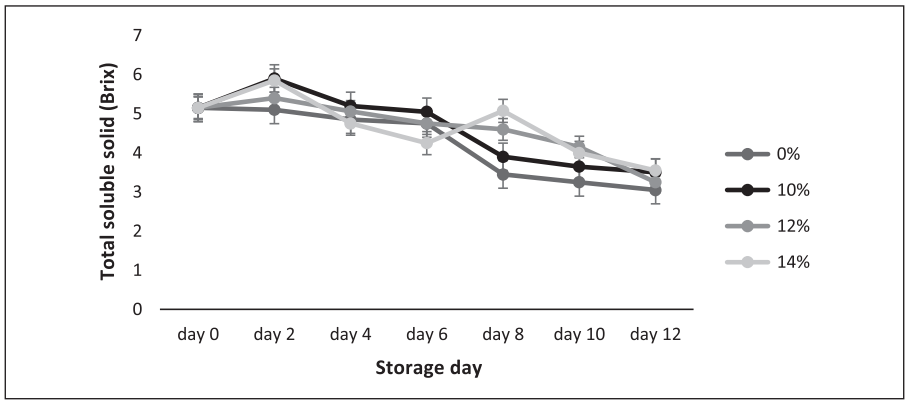


**Fig. 4.** Effect of different water extract propolis concentration as a coating on the soluble solid of strawberry.

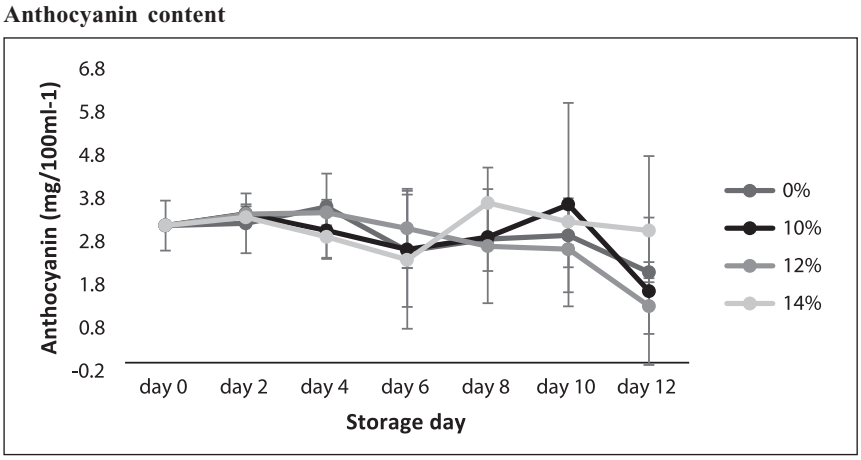


**Fig. 5.** Effect of different water extract propolis concentration as a coating on the anthocyanin content of strawberry.

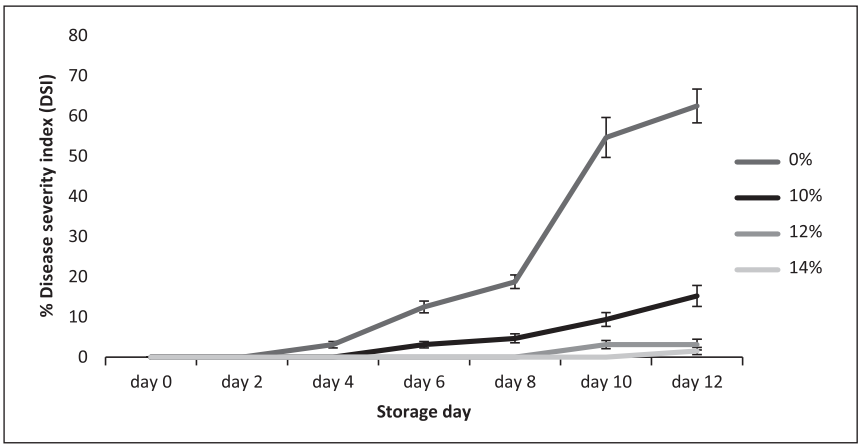


**Fig. 6.** Effect of different water extract propolis concentration on reducing the percentage severity on strawberry.

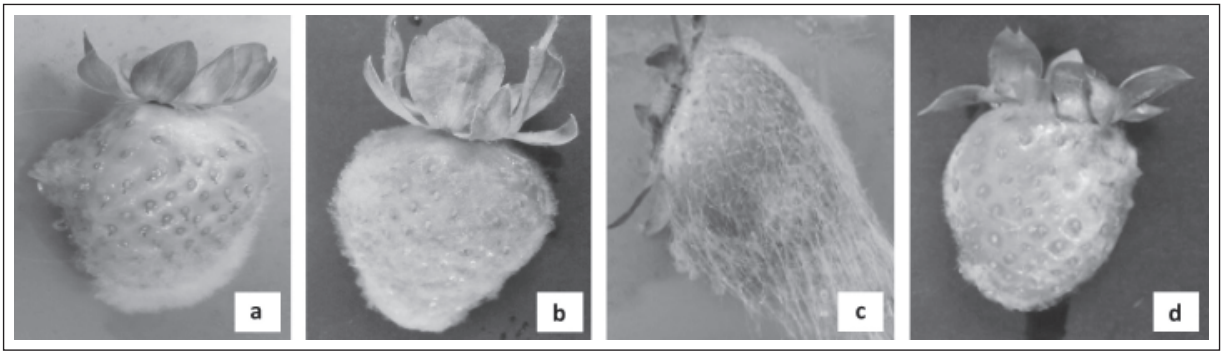


**Fig. 7.** Harvested strawberries deterioration caused by the fungi *Rhizopus stolonifer* with different concentration of WEP (a: 0%, b: 10%, c: 12% and d:14%).

cottony to brownish-black sporangia) which was assumed as *Rhizopus stolonifer* disease (Kwon *et al*., 2009; Feliziani & Romanazzi, 2016). According to Bajpai *et al*. (2010), the existence of *Rhizopus oligosporus* considerably inhibited the growth of the tested pathogens such as *Colletotrichum* sp. Secondary metabolites produced by *Rhizopus oligosporus* may suppress the growth of another fungal pathogen (Jeon *et al*., 2009). However, WEP gives a positive result severity of *Rhizopus*

*stolonifer* disease. Research by Yang *et al*. (2016) demonstrated the powerful inhibitions against spores germination of *Rhizopus stolonifer* by propolis.

## CONCLUSION

This study clearly showed that the propolis did not retain most of the post-harvest quality of infected strawberries except firmness but it successfully reduced the occurrence of *Colletotrichum gloesporioides* by inhibiting mycelium and disease severity. But, this study did not focus on the measurement of ethylene or any parameter that can determine the respiration and ripening, hence any relation of the disease severity on the acceleration of fruit ripening and respiration difficult to be concluded. However, this study can be used as a guideline for future studies on the potential application of propolis as bio-control against *Colletotrichum* sp. Study using the water extract propolis added with other coating materials such as polysaccharide may help in preserving the post-harvest quality of the tested fruits.

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