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Development of chitosan coating product containing bitter gourd extract to delay anthracnose disease in mango

Narueporn BONGKHAM [1], Anupan KONGBUNGKERD [1], Nanthawan HADTHAMARD [2], Maliwan NAKKUNTOD *[1]

[1] Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand.
[2] Faculty of Science and Technology, Kanchanaburi Rajiabhat University, Kanchanaburi, Thailand.
*E-mail: maliwann@nu.ac.th

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ABSTRACT: *Mangifera indica* cv. 'Nam Dok Mai' is the most popular fruit in Asia and worldwide, but it often faces anthracnose disease both in the vegetative stage (on leaves) and the reproductive stage (on fruit). The symptoms of this disease affect low yield and limit export; therefore, the effect of coating to reduce the incidence of anthracnose disease in mango fruits is necessary. This research aimed to develop an edible chitosan coating containing bitter gourd extract. Mango fruits were coated with 0.5, 1.0 and 2.0% chitosan solution (70-75% deacetylation; 310-375 kDa) and appropriate 500 ppm bitter gourd extract. The results showed that the chitosan coating with bitter gourd extract extended the shelf life of mango fruits and decreased the incidence of disease more effectively than coatings with chitosan or bitter gourd extract alone. Further, it could also delay ripening. Disease incidence and lesion diameter on mangoes coated with 2% chitosan with bitter gourd extract were the most effective ($P < 0.05$) within 5, 7 and 10 days after storage. Weight loss in mangoes increases during ripening; however, chitosan coating can help reduce weight loss. Fruits coated with 1% chitosan were the best formula for reducing weight loss. Chitosan coating adding bitter gourd extract is the best choice to replace chemical treatment to obtain better quality before export or transportation.
Keywords: postharvest disease; *Colletotrichum gloeosporioides*; anthracnose fruit injury.

Desenvolvimento de produto de revestimento de quitosana contendo extrato de melão amargo para retardar a antracnose em manga

RESUMO: *Mangifera indica* cv. 'Nam Dok Mai' é a fruta mais popular não só na Ásia, mas também em todo o mundo, mas frequentemente enfrenta uma doença de antracnose tanto no estágio vegetativo (nas folhas), quanto no estágio reprodutivo (na fruta). Os sintomas desta doença afeta o baixo rendimento e limita a exportação, portanto, o efeito do revestimento para reduzir a incidência da doença de antracnose em frutas de manga é necessário. Esta pesquisa teve como objetivo desenvolver um revestimento comestível de quitosana contendo extrato de melão amargo. As frutas de manga foram revestidas com 0,5, 1,0 e 2,0% de solução de quitosana (70-75% de desacetilação; 310-375 kDa) e 500 ppm apropriados de extrato de melão amargo. O resultado mostrou que o revestimento de quitosana com extrato de melão amargonão pode estender a vida útil das frutas de manga e diminuir a incidência da doença por mais tempo quando comparadas com os revestimentos apenas com quitosana ou extrato de melão amargo, e ainda, também pode atrasar o amadurecimento. A incidência de doenças e o diâmetro das lesões nas mangas revestidas com quitosana 2% com extrato de melão amargo foram os mais eficazes ($P < 0,05$) dentro de 5, 7 e 10 dias após o armazenamento. A perda de peso nas mangas aumenta durante o amadurecimento; no entanto, o revestimento de quitosana pode ajudar a reduzir a perda de peso. As frutas revestidas com quitosana 1% foram a melhor fórmula para redução da perda de peso. O revestimento de quitosana adicionando extrato de melão amargo é a melhor escolha para substituir o tratamento químico para obter melhor qualidade antes da exportação ou transporte.
Palavras-chave: doença pós-colheita; *Colletotrichum gloeosporioides*; dano causado por antracnose nos frutos.

1. INTRODUCTION

'Nam Dok Mai' mango (*Mangifera indica* L.) is one of the most important ripe fruits in Thailand. It can be grown in all areas of the region of the country. Most mango products are used for domestic consumption. Still, they are also exported to foreign countries in large quantities because they are economic fruits consumed fresh and processed. In the future, the export volume will likely increase due to its attractive appearance, sweet flavor and juicy flesh; it also has health benefits such as calcium, potassium, vitamin C, vitamin A, vitamin E and vitamin K (MALDONADO-CELIS et al., 2019)

However, mangoes have soft tissue and thin pericarp, including rapid ripening and perishability after harvest, and they are easily infected by anthracnose disease (HE et al., 2016). Several species of *Colletotrichum* cause anthracnose disease, but the species that causes the most serious postharvest disease and severe in mangoes is *Colletotrichum gloeosporioides* (LAKSANAPHISUT et al., 2019). Anthracnose infection causes the produce to be highly perishable and have a short shelf life. This is a limiting factor for exporting mangoes.

This disease management, farmers or gardeners often use chemicals to control and eliminate diseases because chemicals are highly effective in controlling disease and chemical-based coatings are used to delay post-harvest disease and extend shelf life. For example, paraffin and polyethylene wax maintain freshness for a long time and help reduce water loss (KLINSODA, 2016). However, prolonged chemical use results in contamination and the accumulation of chemical residues in the environment; additionally, it leads to drug-resistant fungi and consumer concern about the possible risk of the residue of fungicides. Thus, consumers are giving importance to choosing to consume organic vegetable and fruit products free from toxic substances and chemicals, including methods for extending the shelf life of fruits and vegetables that avoid the use of chemicals by natural compounds.

Chitosan is an alternative approach for extending the shelf life of fruits, their capacity to form non-toxic films and inhibiting the growth of pathogens. Chitosan is the most popular film coating because it is glossy, colorless and antimicrobial (ZHANG et al., 2019). However, high chitosan concentrations often cause fruit ripening problems (JITAREERAT et al., 2007). Chitosan coating has been developed to be combined with others to increase coating efficiency and reduce postharvest disease. The previous study found that the application of chitosan and *Origanum vulgare* L. essential oil in combination can fungicide effects against *Rhizopus stolonifer* and *Aspergillus niger* for reduced the incidence of black mold and soft rot in cherry tomato fruits and drop the water permeability better than use chitosan only (BARRETO et al., 2016). Therefore, natural substances were selected in combination with chitosan to increase the efficiency of inhibiting anthracnose in mango using low concentrations of chitosan.

In the present study, the bitter gourd extract was effective against phytopathogenic fungi (MAHMOOD et al., 2019). The bitter gourd extract was analyzed using various organic solvents revealing the presence of several secondary metabolites, including alkaloids, saponins, tannins and glycosides (CHEONG et al., 2022). The extracts from various parts of the bitter gourd were extracted using water and ethanol to inhibit the germination of spores and the growth of *Fusarium oxysporum* fungal hyphae (GUPTA et al., 2016).

Therefore, this study aimed to develop a chitosan coating containing bitter gourd extract to delay anthracnose's occurrence and prolong the fruit's shelf life for coating solutions without safe chemicals for agriculture, consumers, and the environment.

2. MATERIAL AND METHODS

2.1. Pathogen isolation and identification

Infectious agents were isolated from mango cv. "Nam Dok Mai" is done using the tissue transplanting technique. The regions on the fruit peel showing disease symptoms were cut at the margins of the lesion in dimensions 5x5 mm, dipped in 10% sodium hypochlorite solution for 5 min, and then rinsed with sterilized distilled water 3 times. Each piece was transferred onto Potato Dextrose Agar (PDA) and incubated at 28±3 ºC for 7 days. The mycelia were cultured to the new PDA plate and incubated at 28±3 ºC for 7 days until pure culture per plate. Each pure culture isolation was studied using morphological and molecular data. Each isolate was examined for hypha color and conidia shape for morphological characters under a light microscope. For molecular information, the DNA of each isolate was extracted using the CTAB method. The ITS region was amplified, sequenced, and checked via Blast with the GenBank (NCBI) database for species identification. The fungal mycelium, namely *Colletotrichum gloeosporioide*s, was kept at 20 ºC for further studies.

2.2. Preparation of chitosan coating solution

Fruits of bitter gourd (*Momordica charantia* var. *muricata*) in the mature green stage were obtained from the local market in Phitsanulok, Thailand, during the middle of the year 2022. Fruits were washed with running tap water and cut into small pieces. The fruits were dried in a hot air oven at 45±3 ºC for 5 days and homogenized to a fine powder. The dried powder was immersed and shaken in 95% ethanol for 7 days. Plant extract solutions were filtered using filter paper (Whatman No.1) and evaporated by a rotary evaporator. Crude extracts were freeze-dried to eliminate alcohol. The bitter gourd extract was prepared by dissolving 100% Dimethyl sulfoxide (DMSO) and diluting it with distilled water to 500 ppm for further experimentation.

Chitosan (CHI) of high molecular weight (310-375 kDa; deacetylation degree 75% was obtained from Sigma-Aldrich and dissolved in 1% (v/v) acetic acid (food grade). The solution was stirred to dissolve and incubated at room temperature for 24 hrs. Chitosan solutions were prepared at 0, 0.5, 1.0, and 2% concentrations. After stirring, 500 ppm bitter gourd extract was added to all solutions, and the pH was adjusted to 4.

2.3. Preparation of mango fruits and application of treatment

Mango cv. "Nam Dok Mai" was collected from Phitsanulok province of Thailand in May 2022. Mango fruits at a mature stage of 90-100 days after flowering and mango maturity of 80-85% were selected for uniformity in size and shape and free from disease on fruit peel. Mangoes were washed through running tap water, sterilized in 10% sodium hypochlorite solution for 2 min and rinsed with distilled water. Each fruit was wound with a sterile pipette tip at 2 points, inoculated with fungal spore suspension (5x106) at 10 µL on each wound, and incubated at room temperature for 2 hrs.

After pathogen inoculation, fruits were dipped in 0.5, 1.0 and 2.0% (w/v) chitosan solution and 0.5, 1.0 and 2.0% (w/v) chitosan with 500 ppm of bitter gourd extract and 500 ppm bitter gourd extract only for 1 min. The control fruits were dipped in distilled water. After treatment, the fruits were air-dried at room temperature and stored at 25±3 ºC for 10 days.

Weight loss was measured from initial weight compared with final weight and reported as a percentage. Fruit firmness was measured using a Universal Testing Machine (Instron 5965) with a probe (3-inch diameter) at the pulp 5 mm.

2.4. Data analysis

The anthracnose disease was evaluated on mango fruits with 8 treatments and three replications. After 3, 5, 7, and 10 days of inoculation, anthracnose disease on the fruits was measured in the lesion's length, width and longest diameter. The fruit weight of all treatments was also recorded. Lesion diameter and weight were calculated as the average of replicated treatments. Statistics analysis calculated the mean value of treatment using Duncan's multiple range test. A p-value less than 0.05 was considered statistically significant. Statistical analysis was obtained with IBM SPSS Statistics version 29.0 (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 29.0. Armonk, NY: IBM Corp.)

3. RESULTS

3.1. Pathogen identification

The color of fungal mycelium was white in the initial growth stage at 4 days, then turned to gray hypha when the 7 days finished. The conidia showed a single cell, cylindrical shape with round ends, 14-16 µm in length and 4-5 µm in width (Figure 1). The ITS region of this fungus showed approximate 500 bp and 99.63% identity with *Colletotrichum gloeosporioides* in GenBank database (NCBI) of JX902437.1 and MT012110.1 accession numbers.

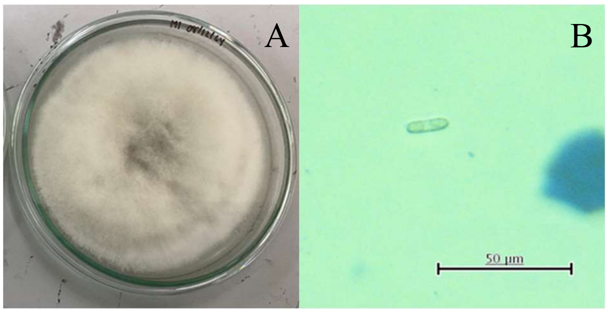

Figure 1. Morphological identification of *Colletotrichum gloeosporioides*, colony color (A) and conidia (B).
Figura 1. Identificação morfológica de *Colletotrichum gloeosporioides*, coloração da colônia (A) e conídios (B).

3.2. Effects of chitosan coating on disease incidence

The coating was divided into 8 treatments, namely control (no coating), only bitter gourd (BT), only chitosan (CHI) in 3 various concentrations (0.5, 1, 2%) and various concentrations of chitosan mixed with 500 ppm bitter gourd extract in reducing the severity of anthracnose disease caused by *Colletotrichum gloeosporioides* on mango fruits was inoculated in the laboratory for 10 days. The results showed that mango fruits presented disease lesions incidence in 3 days of storage in control and BT. The characteristic of the disease is a dark brown spot in the area where the infection was inoculated. However, mangoes in all CHI and 0.5-1% CHI + BT showed symptoms of the disease in 4-5 days of storage, but mangoes coated with 2% CHI + BT had lesions after 5 days. The size of the lesion is larger continuously. In 7 days of storage, the lesion on mangoes coated with 2% CHI + BT had the highest minimum diameter at 1.6 mm. There was a significant difference between the mangoes of all CHI and the control ($P<0.05$). The symptoms showed brown rot at the stem-end of the fruits, and withered fruits were found in control, along with bitter gourd extract treatment (Figure 2-5).

After 7 days of storage until 10 days, the lesions on mango fruits in all CHI and all CHI + BT were significantly lower ($p<0.05$) than in control and BT treatment. The appearance of lesions was the highest in control and BT at 40.2 and 41.8 mm in diameter, respectively, while all treatments of CHI and CHI + BT showed the lesion incidences are less than 20 mm in diameter. It was also found that 2% CHI + BT can reduce the disease severity in mango fruits the most (Figure 6).

3.3. Effects of chitosan coating on disease incidence

Weight loss in the fruits coated with all treatments changed similarly. The weight of fruits would be lost daily after storage if kept at 25 °C. The weight loss of all mango fruits in all treatments stayed at no more than 12%, although the treatment of 1% CHI was the lowest for weight loss at about 9% in 10 days of storage (Figure 7).

Fruits firmness would decrease throughout the storage period. At 3 days after storage, mango fruits in BT treatment showed the lowest fruit firmness, while mango fruits in 1% CHI + BT presented the highest (Figure 8). After 10 days of storage, mango fruits in 1% CHI + BT remained at the highest fruit firmness at 42.45 N, followed by mango fruits coated with 2% CHI, 0.5% CHI + BT, 2% CHI + BT, 1% CHI, 0.5% CHI, BT and control at 38.41, 33.76, 30.26, 19.88, 13.04 and 10.75 N, respectively.

Figure 2. Incidence of disease in mango fruits of control (A), 500 ppm BT (B), 0.5% CHI (C), 1% CHI (D), 2% CHI (E), 0.5% CHI + BT (F), 1% CHI + BT (G) and 2% CHI + BT (H) stored at 25 °C at the day of 3 (BT means bitter gourd extract, CHI means chitosan).
Figura 2. Incidência de doenças em frutos de manga do controle (A), 500 ppm BT (B), 0,5% CHI (C), 1% CHI (D), 2% CHI (E), 0,5% CHI + BT (F), 1% CHI + BT (G) e 2% CHI + BT (H) armazenados a 25 °C no dia 3 (BT significa extrato de melão amargo, CHI significa quitosana)

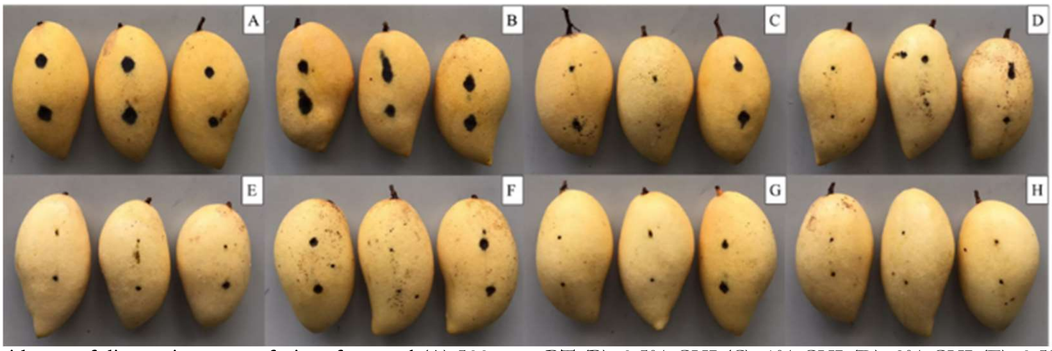

Figure 3. Incidence of disease in mango fruits of control (A),500 ppm BT (B), 0.5% CHI (C), 1% CHI (D), 2% CHI (E), 0.5% CHI + BT (F), 1% CHI + BT (G) and 2% CHI + BT (H) stored at 25 °C at the day of 5 (BT means bitter gourd extract, CHI means chitosan).
Figura 3. Incidência de doenças em frutos de manga do controle (A), 500 ppm BT (B), 0,5% CHI (C), 1% CHI (D), 2% CHI (E), 0,5% CHI + BT (F), 1% CHI + BT (G) e 2% CHI + BT (H) armazenados a 25 °C no dia 5 (BT significa extrato de melão amargo, CHI significa quitosana).

Figure 4. Incidence of disease in mango fruits of control (A), 500 ppm BT (B), 0.5% CHI (C), 1% CHI (D), 2% CHI (E), 0.5% CHI + BT (F), 1% CHI + BT (G) and 2% CHI + BT (H) stored at 25 °C at the day of 7 (BT means bitter gourd extract, CHI means chitosan).
Figura 4. Incidência de doenças em frutos de manga do controle (A), 500 ppm BT (B), 0,5% CHI (C), 1% CHI (D), 2% CHI (E), 0,5% CHI + BT (F), 1% CHI + BT (G) e 2% CHI + BT (H) armazenados a 25 °C no dia 7 (BT significa extrato de melão amargo, CHI significa quitosana).

Figure 5. Incidence of disease in mango fruits of control (A), 500 ppm BT (B), 0.5% CHI (C), 1% CHI (D), 2% CHI (E), 0.5% CHI + BT (F), 1% CHI + BT (G) and 2% CHI + BT (H) stored at 25 °C at the day of 10 (BT means bitter gourd extract, CHI means chitosan).
Figura 5. Incidência de doenças em frutos de manga controle (A), 500 ppm BT (B), 0,5% CHI (C), 1% CHI (D), 2% CHI (E), 0,5% CHI + BT (F), 1% CHI + BT (G) e 2% CHI + BT (H) armazenados a 25 °C no dia 10 (BT significa extrato de melão amargo, CHI significa quitosana).

4. DISCUSSION

For fungal identification, morphological characteristics of the mycelium of *C. gloeosporioides* on PDA medium presented greyish white and dark grey at the point of inoculation. The result is similar to the earlier description by Khanzada et al. (2018). Moreover, Aruna Prasad et al. (2022) reported that the mycelium of *C. gloeosporioides* showed dense, cottony, and white to greyish; the conidia were cylindrical with both round ends, measuring 14-16 μm in length and 4-6 μm width. The nrITS region is considered the universal barcode of fungal identification (BADOTTI et al., 2017).

In this study, only chitosan and combining chitosan and bitter gourd extract can reduce anthracnose disease incidence. The 2% Chitosan + BT coating is the best formula for reducing disease incidence. This showed that both chitosan and substances in bitter gourd extract synergize. In general, chitosan coating is a natural compound used to shift the life extension of fruits and maintain quality attributes postharvest because of its film-forming and antifungal properties (KUMARIHAMI et al., 2021). Moreover, many research reported that chitosan has antifungal activity that can inhibit the growth of fungal mycelia such as *Aspergillus ochraceus* (Meng et al., 2020), *Fusarium oxysporum* (Dananjaya et al., 2017) and *Colletotrichum gloeosporioides* (BAUTISTA-BAÑOS et al., 2006).

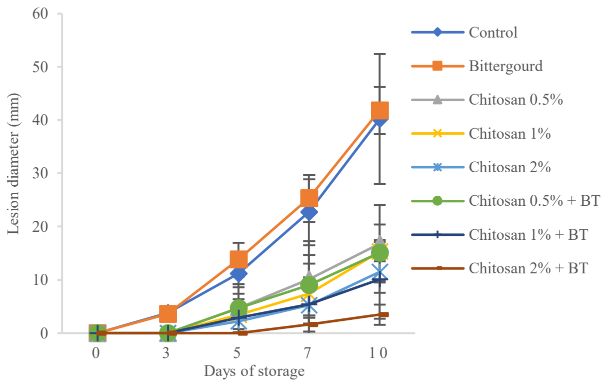

Figure 6. Effect of chitosan on lesion average diameter in mango fruits inoculated with *Colletotrichum gloeosporioides* after incubation at 25 °C for 10 days. Vertical bars represent the standard derivation of mean (n=6).
Figura 6. Efeito da quitosana no diâmetro médio da lesão em frutos de manga inoculados com *Colletotrichum gloeosporioides* após incubação a 25 °C por 10 dias. Barras verticais representam a derivação padrão da média (n=6).

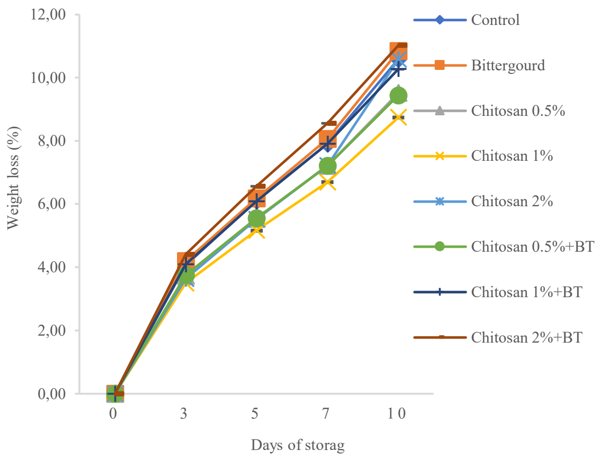

Figure 7. Effect of chitosan coating and chitosan with bitter gourd extract on the loss of weight of mango after storage at 25 °C for 10 days. Vertical bars represent the standard derivation of mean (n=3).
Figura 7. Efeito do revestimento de quitosana e quitosana com extrato de melão amargo na perda de peso da manga após armazenamento a 25 °C por 10 dias. Barras verticais representam a derivação padrão da média (n=3).

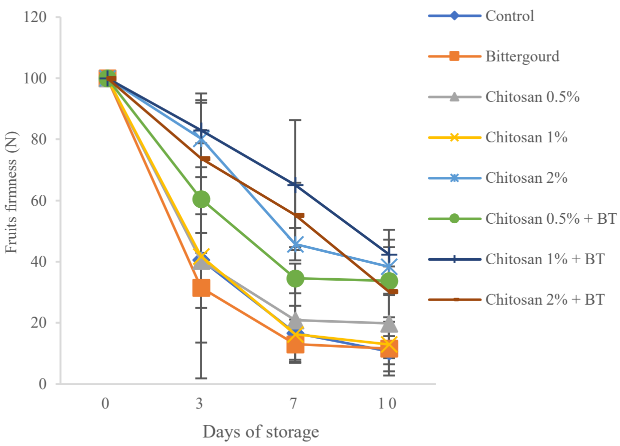

Figure 8. Changes in fruit firmness of mango fruits coated with chitosan and chitosan+BT during storage at 25 ºC for 10 days. Vertical bars represent the standard derivation of mean (n=3).
Figura 8. Mudanças na firmeza dos frutos de manga revestidos com quitosana e quitosana+BT durante o armazenamento a 25 ºC por 10 dias. Barras verticais representam a derivação padrão da média (n=3).

The effect of chitosan on fungi was mentioned, and it plays a major role in hypha growth, preventing and inhibiting spore germination (HASSAN; CHANG, 2017). The important compounds in bitter gourd extract are phytochemical flavonoids, saponins and phenolic compounds (MEDEIROS et al., 2020). These substances have been pointed out to inhibit the growth of the fungus, namely *C. gloeosporioides,* which causes anthracnose (VÁZQUEZ-GONZÁLEZ et al., 2020).

Extracts from various parts of bitter gourd extracted with water and ethanol could inhibit fungal growth and the spore germination of *Alternaria alternata* (GUPTA et al., 2017). Furthermore, Gusmão et al. (2018) reported that bitter gourd extract effectively inhibited the spore germination of *C. gloeosporiodes.* They effectively reduced the severity of anthracnose lesions in inflorescences of parrot heliconia (*Heliconia psittacorum* L.f.). However, our results showed that if only bitter gourd extract were applied to mango fruits, it could not decrease the severity of anthracnose disease. If only chitosan were used as the coating on mango, the disease lesion would still occur. Thus, the combination of both should be considered. From our study, the combination was better used than the single application.

The synergism between chitosan and biochemical substances in bitter gourd has never been previously reported. Still, the combination of chitosan and other chemicals, such as copper nanoparticles, has been reported, maintaining the antifungal activity and potentially a significant step towards more sustainable agriculture (LEMKE et al., 2022).

When mango fruits begin the ripening stage, the texture softens, weight loss increases, and firmness decreases. Our study showed that mango fruits coated with chitosan only have a lower percentage of weight loss than mango fruits coated with chitosan with bitter gourd extract. Using chitosan and combining chitosan and bitter gourd extract can delay ripening because the coating may form a layer film barrier on the skin surface and retain firmness.

Thereby, the respiration rate of the fruit slows down because the chitosan coating on mangoes forms a film on the surface of the fruit to prevent water loss (KUMAR et al., 2017) and O_2 that causes reduced respiration rate of the fruits, resulting in mangoes is prolong the postharvest shelf life of the mangoes (CAMATARI et al., 2018). However, in our study, mango fruits coated with 2% chitosan + 500 ppm of bitter gourd have the highest percentage of weight loss because 2% chitosan is a high concentration, so the solution is viscosity and when coated with the chitosan on fruit, it film-firming is thicker. Therefore, when the coating was dry, the chitosan film was cracked and peeled off the fruits, so mango fruits had more respiration and weight loss than the fruit coated with a lower concentration of chitosan, which caused mango to ripen faster. Weight loss influences fruit quality attributes and limits postharvest storage (VIVEK; SUBBARAO, 2018).

Our study showed that mago fruits coated with 1% chitosan had lower firmness than mango fruits coated with all the combinations between chitosan and bitter gourd extract because 1% chitosan is a high concentration, and chitosan in this study has a high molecular weight, so it has a lower rate of respiration and ethylene production (Jitareerat et al. 2007). Thus, the high concentrations of chitosan also resulted in abnormal ripening of mangoes.

5. CONCLUSIONS

The development of a chitosan coating solution product containing bitter gourd extract, it was found that 2% chitosan containing 500 ppm of bitter gourd extract is the optimal concentration to reduce anthracnose disease. In comparison, 1% chitosan containing 500 ppm bitter gourd extract coating on mango fruits could delay the ripening process by inhibiting the respiration rate in the mango fruits. However, it is useful in coating the fruit surface as an alternative approach for practice use in mangoes.

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