Control of postharvest fungal decay in papaya fruits by chitosan-carboxymethyl cellulose coating incorporated with essential oils and potassium sorbate

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ABSTRACT: The objective of this study was to evaluate the effect of plant essential oils in combination with acid salts for development of edible coating formulations. Firstly, antifungal activities of cinnamon (*Cinnamomum verum*), clove (*Syzygium aromaticum*), and colla aromatica Roxb. (*Homalomena aromatica*) oils and acid salts (ammonium carbonate, potassium metabisulfite, and potassium sorbate) were studied. Cinnamon and clove oils at 0.0125–0.2% minimum inhibitory concentration (MIC) were effective to inhibit growth of *Aspergillus flavus* TISTR 3041, *Penicillium citrinum* TISTR 3437, and 2 mold strains, *Talaromyces* sp. P2D4 and *Aspergillus* sp. P8A6 isolated from papaya fruits, while those salts showed antifungal activity at 0.5–8% MIC. Combination of cinnamon or clove oil with potassium sorbate showed the synergistic effect against *Talaromyces* sp. P2D4. Effect of cinnamon or clove oil combined with potassium sorbate in 0.1% chitosan and 0.25% carboxymethyl cellulose (ChiCMC) coating on controlling postharvest fungal decay in papaya fruits during storage at 30 °C for 8 days was studied. Synergistic combinations of 0.0397% clove oil combined with 0.25% potassium sorbate, or 0.25% cinnamon oil combined with 1% potassium sorbate in the ChiCMC coating were effective to delay fungal decay in papaya fruits. At the end of storage, these coatings significantly showed less decay incidence (53.3–56.7%) compared to a control treatment (100% decay incidence). Thus, use of cinnamon or clove oil combined with potassium sorbate in the ChiCMC coating could potentially be used in the postharvest management for decay control in papaya fruits.

KEYWORDS: clove oil, cinnamon oil, potassium sorbate, *Talaromyces*, *Aspergillus*

INTRODUCTION

Papaya fruits (*Carica papaya* L.) have a short postharvest life. Rapid deterioration of papaya fruits is due to their nutritional characteristics and can be originated from their high respiration rate and other biochemical transformations. These can promote growth of microorganisms contaminated during postharvest or storage event, resulting in development of postharvest diseases. Fungi are the main cause of the papaya rot disease. Several molds associated with spoiled papaya fruits include *Penicillium digitatum* (penicillium rot), *Collectotrichum gloeosporiodes* (anthracnose), *Rhizopus stolonifer* (rhizopus soft rot), *Alternaria solani* (alternaria fruit spot), *Fusarium* spp., *Mucor* spp., and *Aspergillus* spp. [1]. Control of fungal decay in papaya fruits can be performed in several ways including heat treatment, irradiation, modified atmosphere, and chemical control using synthetic fungicides such as thiabendazole, chlorothalonil, prochloraz, azoxystrobin, fludioxonil, and imazalil. Chemical treatment is a convenient and rapid way, but it depends on the amount of chemical residue on fruits. Alternative methods of using natural products and low residue chemicals have been suggested for retardation of papaya spoilage [2]. One of the possible alternative treatments is using plant essential oils and acid salts for controlling postharvest fungal diseases. Essential oils consist of a variety of volatile active substances and can be synthesized from all plant parts including buds, flowers, leaves, stems, fruits, wood, and bark. Plant essential oils have been reported to inhibit the growth of fungi causing spoilage of agricultural produce [3]. Several researchers reported the effective antifungal activity of cinnamon and clove oils with medicinal properties such as anti-inflammatory, antiseptic, etc [4]. Nevertheless, few studies have been reported on the antifungal activity of *Homalomena aromatica* (colla aromatic Roxb.).

Essential oils have been used as an alternative postharvest treatment to control diseases of fresh agricultural produces. Some researchers have studied the incorporation of cinnamon and clove oils in edible coating to delay spoilage of fruits such as papaya, banana, strawberry, blueberry, apple, orange, etc [5]. Besides, other preservatives such as organic and inorganic acids and their salts can be used as fungicides to control postharvest diseases of agricultural produce. Acid salts have been used to incorporate into edible coating for controlling of postharvest fungal diseases [6].

Edible coating, a biopolymer contains several active ingredients protecting foods from physical, chemical, and biological degradation and has been reported to decrease microbial growth, prevent the physical damage, and protect the loss of volatile com-

pounds in fruits. Several studies have shown that the edible coating containing polysaccharide could enhance the shelf life and quality of fresh agricultural produce [6]. Chitosan and carboxymethyl cellulose (CMC) are biodegradable, nontoxic, and inexpensive. These biopolymers can be used to prevent physiological loss in weight and microbial proliferation in agricultural produce after harvesting [7].

Chitosan is a deacetylated amino polysaccharide of chitin extensively used in food industries due to its less toxic and biodegradable properties [8]. Edible coating that contains only chitosan has some disadvantages such as its high permeability to water vapor and weak mechanical property [7]. This can affect instability of the coating, and it tends to peel off the surface of agriculture produce during storage. The quality of the chitosan coating should be improved by mixing with other types of polysaccharide materials such as CMC. CMC is a water-soluble cellulose derivative, containing glucopyranosyl units with a high molecular weight. These provide structural integrity and strength of the edible coating [6]. Thus, a transparent film with good appearance and barrier property can be made by mixing CMC with chitosan [9]. Chitosan mixed with CMC has been used in edible coating for citrus fruits [10]. Plant essential oils and acid salts have been used to incorporate into chitosan or CMC to produce edible coating for controlling fungi in papaya fruits [6], but combined effect of the essential oil and acid salt in edible coating has not been reported. Thus, this study evaluated whether the edible coating of mixed chitosan and CMC in combination with the essential oil and acid salt could effectively control postharvest fungal decay in papaya fruits.

MATERIALS AND METHODS

Plant materials

Dried medicinal plants of cinnamon (*Cinnamomum verum* stem bark), clove (*Syzygium aromaticum* flowers), and colla aromatica Roxb. (*Homalomena aromatica* Schott. rhizomes) were purchased at the local market in Ladkrabang District, Bangkok, Thailand. Fresh papaya fruits (*Carica papaya* L., Holland papaya variety) were purchased from a wholesale market (Talaad Thai) in Pathum Thani Province, Thailand.

Preparation of essential oils

All dried medicinal plants were ground and extracted by hydrodistillation process using a Clevenger's apparatus. Briefly, 150 g medicinal plant powder were placed in the distillation flask. Then, water was added and heated for 3–4 h until the amount of the oil in the receiver was steady. Sodium sulfate anhydrous was added and stirred to remove the excess water before collecting the oil.

Fungal strains and inoculum preparation

Two reference mold strains, *A. flavus* TISTR 3041 and *P. citrinum* TISTR 3437, obtained from the Microbiological Resources Centre for Southeast Asian Region (Bangkok MIRCEN), Thailand, were grown on Potato Dextrose Agar (PDA) at 30 °C for 7 days. To prepare spore suspension, 3 ml of 0.1% (v/v) tween 80 was poured into each culture tube, and a sterile Pasteur pipette was used for scraping the agar surface to release the spores. Then, the mycelium was filtered through a sterile cotton wool for spore collection. The number of spores was counted using hemacytometer and adjusted to 10^6 spores/ml with 0.1% tween 80.

Study of fungal contamination in papaya

In this study, papaya fruits (10 samples) were evaluated for fungal contamination by dilution plating method [11]. The samples were plated onto acidified PDA and Dichloran Rose Bengal Chloramphenicol Agar (DRBC). The measurement of pH and water activity of all samples was performed by pH meter (Testo 205 AG, Germany) and water activity meter (AquaLab Series 3TE, Decagon Devices, Inc., USA), respectively.

Isolation and identification of fungi

The morphological characteristics of fungal colonies grown on acidified PDA and DRBC were observed. Then, each different colony was transferred to 2 agar media including Czapek Yeast Extract Agar (CYA) and Malt Extract Agar (MEA), incubated at 30 °C for 7 days for molds and 3 days for yeasts, and re-isolated until getting pure cultures. All mold and yeast isolates were maintained on PDA and Yeast Malt Agar (YMA), respectively. Yeast isolates were identified by using miniaturized biochemical test kit (API 20 C AUX, bioMérieux, France). Morphological characteristics of all mold isolates were observed using the procedure as described by Samson et al [12]. The identification of 2 molds isolated from papaya fruits was performed by molecular methods.

Strain identification by ITS1-ITS4 sequencing analysis

The fungal isolates were cultured on PDA and incubated at 30 °C for 7 days. Their spores were collected and adjusted to 10^6 spores/ml concentration with 0.1% (v/v) tween 80. Genomic DNA of each mold was isolated from spore suspension using FavorPrep™ Fungi/Yeast genomic DNA extraction mini kit (Flavorgen, Taiwan). PCR amplification of the highly variable ITS1 and ITS2 sequences surrounding the sequence of 5.8S rDNA and situating between the small subunit-coding sequence and the large subunit-coding sequence was carried out using universal primers: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [13]. Amplification was performed in a 50-µl reaction containing KAPA *Taq*

ready mix (Sigma, USA) with 50 ng of genomic DNA, 0.25 µM ITS1, and 0.25 µM ITS4. The PCR conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, primer extension at 72 °C for 90 s, and finally final extension at 72 °C for 10 min. The PCR products were purified by PCR cleanup kit (Geneaid, Taiwan). The purified PCR products were sequenced by U2Bio company (Thailand). The obtained nucleotide sequences were compared with other sequences from Genbank databases using the BLASTn (Nucleotide Basic local alignment search tool) program [14]. The evolutionary relationship among fungal strains was analyzed by constructing a phylogenetic tree of ITS1-ITS4 with maximum likelihood (ML) method using MEGA11 (Molecular Evolutionary Genetics Analysis) software [15]. Bootstrap value was obtained from 1,000 replicates for each database.

Determination of minimum inhibitory concentration (MIC) of essential oils

The MIC test was performed against the 2 reference mold strains, *A. flavus* TISTR 3041 and *P. citrinum* TISTR 3437 and 2 selected molds isolated from papaya fruits using agar dilution method as described by Collins et al [16] with minor modification. Firstly, the molten PDA mixed with stock solution of each sample in 10% (v/v) dimethyl sulfoxide (DMSO) was prepared to obtain a final concentration ranging from 0.0063–0.8% (v/v) for cinnamon, clove, and colla aromatic Roxb. oils. In this study, 50–5,000 ppm chlorothalonil, 0.0076–30.266 mM cinnamaldehyde, 0.0062–24.360 mM eugenol, and 0.0064–25.932 mM linalool were used as a positive control. Then, the medium surface was left to dry before 5 µl of the spore suspension at 10^6 spores/ml was inoculated. After incubation at 30 °C for 7 days, each plate was checked for the presence or absence of fungal colonies. The MIC was evaluated from the lowest concentration of the sample that could inhibit the growth of the inoculated mold. DMSO at 10% (v/v) was used as a negative control. All tests were performed in triplicate.

Determination of minimum inhibitory concentration of acid salts

The MICs of 3 acid salts including ammonium carbonate, potassium metabisulfite, and potassium sorbate were determined using the same method as described above, excepting that a filter sterilized acid salt at 5–100% was used to dilute with sterile distilled water and molten PDA to obtain a final concentration ranging from 0.01–10%. After inoculation and incubation, each plate was checked for the presence or absence of fungal colonies [17]. Then, the MIC value was determined.

Synergy testing of mixed essential oils and acid salts

Synergy assay was performed by agar dilution checkerboard method as described by Rosato et al [18]. The combinations of i) cinnamon oil and potassium sorbate, ii) clove oil and potassium sorbate, iii) cinnamon oil and ammonium carbonate, and iv) clove oil and ammonium carbonate were evaluated for their antifungal activity against a selected mold strain isolated from papaya. To do this test, each oil and acid salt combination was prepared at different concentrations (1/2, 1/4, 1/8, and 1/16 of their MIC values). In each combination, a total volume of 1 ml containing 0.5 ml of the essential oil and 0.5 ml of the acid salt was mixed with 19 ml of molten PDA, left to solidify until surface was dried. Then, 5 µl of mold spore suspension (10^6 spores/ml) were inoculated at the center of the agar plate, and the plate was incubated at 30 °C for 7 days. The fractional inhibitory concentration index (FICI) was calculated [19].

Antifungal effect of essential oils in combination with acid salts on mycelium growth

Inhibitory effect of essential oils in combination with acid salts on mycelium growth of a selected mold isolated from the papaya fruit was assessed using the poison agar method described by Ali et al [20]. The selected essential oils at 0.0397–0.25% (v/v) and acid salts at 0.125–1.0% (v/v) (either alone or combination) were incorporated into PDA. Sterile distilled water was used as a negative control. One agar plug (5 mm diameter) of a 7-day old mold was transferred to the midpoint of the plate and incubated at 30 °C for 7 days. Then, diameter of radial mycelial growth was measured. The percentage of mycelial growth inhibition (% MGI) was calculated using the formula: $\%\,MGI = [C - T/C] \times 100$, where C is the diameter of mycelial growth on negative control PDA, and T is the diameter of mycelial growth on PDA supplemented with each concentration of the plant oil and acid salt or their combinations.

Effect of ChiCMC edible coating incorporated with essential oils and acid salts on physicochemical parameters of papaya fruits inoculated with the selected mold isolate

Fruit preparation

Papaya fruits (Holland variety) with index maturity stage 2 (green with slightly yellow color) were washed with tap water. The fruit surface was then sterilized by soaking in 0.5% (v/v) sodium hypochlorite for 2 min, then rinsed with sterile distilled water, and allowed to air dry at room temperature for 1 h.

Preparation of edible coating

Edible coating was prepared as described by Noshirvani et al [21] with some modifications. Chitosan at

0.2% (w/v) and CMC at 0.5% (w/v) were prepared separately. Chitosan powder (Food grade, particle size of 200 mesh, and molecular weight of 8.97×10^5 Dalton, Sinudom Agriculture Product Limited Partnership, Thailand) was dispersed in aqueous solution of 0.5% (v/v) glacial acetic acid. The mixture was stirred overnight at ambient temperature. The CMC powder (Chawaree Protrade Co., Ltd., Thailand) was dissolved by mixing thoroughly in distilled water and left for 3 h until it was completely dissolved. These solutions at the same volume were mixed for 1 min using a food mixer (Sokany, China) to get the mixture of chitosan and CMC (ChiCMC) at final concentration of 0.1% (w/v) chitosan and 0.25% (w/v) CMC. Then, the essential oil and acid salt at each concentration as well as 0.2% (v/v) tween 80 and 2.5% (v/v) glycerol were added into ChiCMC solution and finally homogenized thoroughly using the food mixer for 30 s. The pH value was adjusted to 6.8 with 3 M NaOH. Six formulations of edible coating were prepared as follows: T1, sterile water as control; T2, ChiCMC; T3, ChiCMC mixed with 7,500 ppm chlorothalonil (CTN); T4, ChiCMC mixed with 0.0397% clove oil and 0.25% potassium sorbate (PS); T5, ChiCMC mixed with 0.0397% cinnamon oil and 0.25% PS, and T6, ChiCMC mixed with 0.25% cinnamon oil and 1% PS.

Papaya treatments

Treatments of papaya fruits were performed using the method as described by Gomes et al [22]. Three wounds were done on a sterilized papaya fruit surface using a sterile cork borer (4 mm diameter and 3 mm deep). Then, all fruits were divided into 6 groups (9 fruits each). Fruits of each group were brushed with each edible coating and left for surface drying. Spore suspension of a selected mold isolate (50 µl of 10^6 spores/ml) was inoculated into each wound. Each fruit was placed on a sterile disposable tray lined with moistened paper towel and packed into a sterile plastic bag. All fruits were stored at 30 °C at 90% relative humidity for 8 days. The percentage of disease control efficiency, total soluble solid, titratable acidity, pH value, percentage of weight loss, and mature index were determined at day 0, 4, and 8 of storage. In addition, the effect of all ChiCMC coatings on membrane integrity of the selected mold isolate was also performed.

a) Determination of disease control efficiency

To determine the percentage of disease control efficiency (% DCE), the number of lesions in each fruit was counted, and the diameter of each lesion was measured. The % DCE was calculated as the following formula: DCE (%) = $[(A-B)\times100]/A$, where A is lesion diameter in a negative control fruit and B is lesion diameter in a treated fruit.

b) Determination of total soluble solid

To determine total soluble solid (TSS), papaya fruit pulp of each sample was squeezed to get papaya juice and measured for its TSS using a refractometer.

c) Determination of titratable acidity

The titratable acidity of papaya fruit pulp was determined according to the method as described by Ali et al [20] with some modifications. Briefly, 10 g papaya fruit pulp was added with 40 ml of distilled water and blended by a fruit mixer. After filtering, 20 ml of papaya juice was used to analyze the titratable acidity (TA). The TA (%) was calculated as citric acid equivalent using the following formula: % TA = $[A \times B \times C \times D \times 100]/[E \times F \times 1000]$, where A is ml of NaOH used for titration, B is concentration (molar) of NaOH (0.1 M), C is total volume of juice, D is equivalent weight of citric acid (64), E is weight of papaya fruit pulp (10 g), and F is volume of juice used for titration (20 ml).

d) Determination of pH value

To determine pH value, the papaya pulp of each treatment was ground. Then, the pH value of each sample was measured using a portable pH meter (Testo 205, Testo AG, Germany).

e) Determination of weight loss

Papaya fruits of each treatment were weighed at the beginning of storage. After storage for 4 and 8 days, the same papaya fruit was taken out, weighed, and then returned into its packaging. Percentage of weight loss was calculated using the following formula: % Weight loss = $[A - B/A] \times 100$, where A is initial weight (g) and B is final weight (g) at each storage time interval.

f) Determination of mature index

Mature index (MI) was calculated as described by Peralta-Ruiz et al [23]. To determine the mature index of each papaya fruit, its total soluble solid (° brix) was divided by its total acidity.

g) Effect of edible coating on membrane integrity of the selected mold

The effect of edible coating on cytoplasmic membrane damage was evidenced by Evans blue staining according to Peralta-Ruiz et al [23] with slight modification. For slide culture preparation of the selected mold, spore suspension (30 µl) of 7-day old mold was placed on a sterile coverslip containing 100 µl of Potato Dextrose Broth (PDB). After 7 days of incubation at 30 °C, each edible coating solution (500 µl) was added to the hyphae attached to the coverslip and left for 6 h. After removing the coating solution, hyphae were stained with 1% Evans blue (ACROS ORGANICS, USA) in Phosphate Buffer Saline (PBS) for 5 min. After staining, the excess dye was removed by washing with

PBS. The hyphae were observed under a bright-field microscope.

Effect of ChiCMC edible coating incorporated with essential oils and potassium sorbate on decay incidence of non-inoculated papaya fruits

The surface of all papaya fruits was sterilized by dipping in 0.5% (v/v) sodium hypochlorite and then rinsed with sterile water. After surface drying, papaya fruits were divided into 6 groups (10 fruits each) and coated with each coating solution (the same coating used for the inoculated fruits). The surface of all papaya fruits was dried. All fruits were packed and stored in the same condition with inoculated fruits. Papaya fruit decay was assessed visually for rot appearance after 8 days of storage. The percentage of decay incidence was calculated.

Statistical analysis

Data of 3 replications were used to perform statistical analysis using analysis of variance and Duncan's multiple range test to compare whether significant difference existed between treatment mean at 95% confident limit using SPSS statistical package version 26 (MBI, USA).

RESULTS

Fungal contamination in papaya

The number of total yeast and mold counts from ripe papaya fruits was in the range of 1.0×10^5–2.3×10^8 CFU/g on acidified PDA and 8.0×10^5–2.4×10^8 CFU/g on DRBC. The most common mold isolates were *Penicillium* spp. (58.34%), and a few others were found (8.34% each) including *Absidia* spp., *Acremonium* spp., *Aspergillus* spp., *Paecilomyces* spp., and a yeast-like fungus including *Trichosporon* spp. These papaya fruits had an average pH and a_w of 5.56 and 0.98, respectively. Among the obtained mold isolates, the isolates P2D4 and P8A6 resemble the characteristics of *Penicillium* and *Aspergillus*, respectively. Thus, they were selected since these molds are the common fungi usually associated with spoiled papaya fruits according to Bautista-Baños [1].

Morphological and molecular identification of the two selected mold isolates

The identification of the mold isolates P2D4 and P8A6 was investigated by morphological and molecular characterization analysis. The colony characteristic of the isolate P2D4 on PDA showed that at 30 °C, a colony diameter reached 5.0–5.2 cm within 7 days, whereas at 25 °C, it was slightly smaller with a colony diameter of 4.6–4.8 cm. The colony exhibited a raised elevation at the point of inoculation. The sporulation was moderately dense and formed compact chains. A soluble pigment with an orange-red color was observed (Fig. 1A-C), while the mycelia appeared white. The conidiophores were biverticillate with smooth walls, and 3–5 metulae were present. The phialides exhibited an acerose form, and the conidia were ellipsoidal in shape with smooth walls (Fig. 1D). By the phylogenetic tree based on ITS1–ITS4 sequencing using maximum likelihood method, ITS1–ITS4 sequences of the isolate P2D4 were closely related to those of *Talaromyces* sp. (Fig. 1G). *Talaromyces* is a teleomorph of *Penicillium*. Therefore, the isolate P2D4 was renamed as *Talaromyces* sp. P2D4.

The 7-day old of *Aspergillus* sp. P8A6 from a moldy papaya grown on PDA attained a colony with diameter of 7.1–7.2 cm at 30 °C (5.0–5.3 cm at 25 °C). Its conidia were yellow green in color, globose, and smooth-walled, while conidiophores were rough-walled with vesicle subglobose with 4 phialides (Fig. 2A-C). Based on ITS1–ITS4 phylogenetic tree analysis using Maximum Likelihood method, *Aspergillus* sp. P8A6 was grouped in the same cluster with *Aspergillus oryzae* NRRL 447 and was closely related to other species of *Aspergillus* (Fig. 2F), confirming that isolate P8A6 belonged to *Aspergillus* species.

### Antifungal activity of essential oils

Cinnamon, clove, and colla aromatica Roxb. oils could inhibit the growth of all fungal strains tested. Among these oils, cinnamon oil displayed the strongest antifungal activity with 0.0125–0.025% MIC, followed by clove oil with 0.05–0.2% MIC and colla aromatic Roxb. oil with 0.2–0.8% MIC. Among all compounds, cinnamaldehyde was the most potent compound to inhibit *Talaromyces* sp. P2D4. This mold was the most sensitive to be inhibited by the positive control chlorothalonil with 50 ppm MIC (Table 1). Therefore, cinnamon and clove oils were selected for application in production of edible coating.

Antifungal activity of acid salts

Studying of antifungal effect revealed that ammonium carbonate showed the most effective salt to inhibit growth of *Aspergillus* sp. P8A6, *A. flavus* TISTR 3041, and *P. citrinum* TISTR 3437 at 0.5% MIC. Potassium sorbate showed highly effective inhibition on growth of *Talaromyces* sp. P2D4 with 0.5% MIC as compared to other mold strains, while potassium metabisulfite possessed moderate antifungal activity against all mold strains tested with 1–1.5% MIC. However, *Talaromyces* sp. P2D4 was resistant to sodium benzoate, and *Aspergillus* sp. P8A6 was resistant to potassium sorbate (Table 1).

Synergy effect of essential oils and acid salts on fungal inhibition

Synergy assay of combined cinnamon or clove oils with potassium sorbate against *Talaromyces* sp. P2D4 showed partial synergism or synergism with FICI of 0.5–0.75, but no synergy effect was found when tested
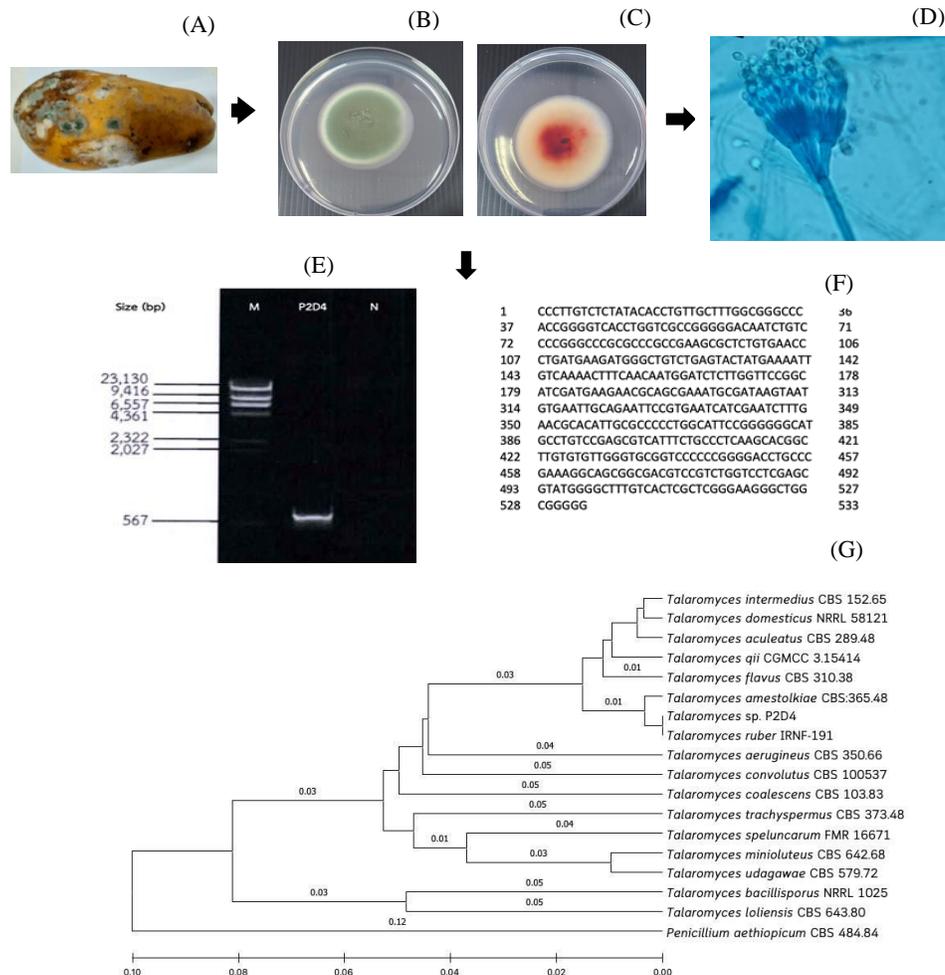
Fig. 1 Morphological and molecular identification of the isolate P2D4: (A) a moldy papaya; (B) morphology of the isolate P2D4 on PDA at 30 °C, 7 days; (C) reverse of (B); (D) microscopic observation (400 ×); (E) Agarose gel electrophoresis of the PCR products with ITS1–ITS4 target sequences: Lane 1, DNA marker λ/*Hind*III; Lane 2, amplified PCR products; and Lane 3, negative control; (F) DNA sequence of ITS1–ITS4 fragment of PCR products; and (G) phylogenetic tree analysis based on ITS1–ITS4 using Maximum Likelihood method with 1,000 bootstraps.

with these oils combined with ammonium carbonate (Table 2). Thus, the combination of either cinnamon or clove oil with potassium sorbate was selected to apply in edible coating.

### Effect of essential oils and acid salts on mycelial growth inhibition

In this study, cinnamon oil at 0.0397 and 0.0625% showed 61.23 and 93.34% MGI against *Talaromyces* sp. P2D4, respectively. Clove oil at 0.0397% displayed 61.74% MGI, but 0.125–0.25% cinnamon oil and 0.0625–0.25% clove oil could completely inhibit mycelial growth of *Talaromyces* sp. P2D4. Ammonium carbonate and potassium sorbate at 0.125–1.0% showed 38.78–79.17% and 5.1–62.75 % MGI, respectively. Study of the combined effect of essential oils and

acid salts revealed that all combinations of cinnamon oil (0.0397–0.25%) and potassium sorbate (0.125–1.0%) exhibited 100% mycelium growth inhibition of *Talaromyces* sp. P2D4. Therefore, the combination of clove or cinnamon oil (0.0397–0.25%) with potassium sorbate (0.25–1.0%) was selected to develop the edible coating formulations.

Combined effect of essential oils and acid salts in ChiCMC edible coating on fungal decay and physicochemical parameters of papaya fruits

a) Disease control efficacy (DCE)

ChiCMC incorporated with cinnamon or clove oil and potassium sorbate showed 50–70% DCE of papaya fruits after 4 days of storage at 30 °C (Fig. 3A). After
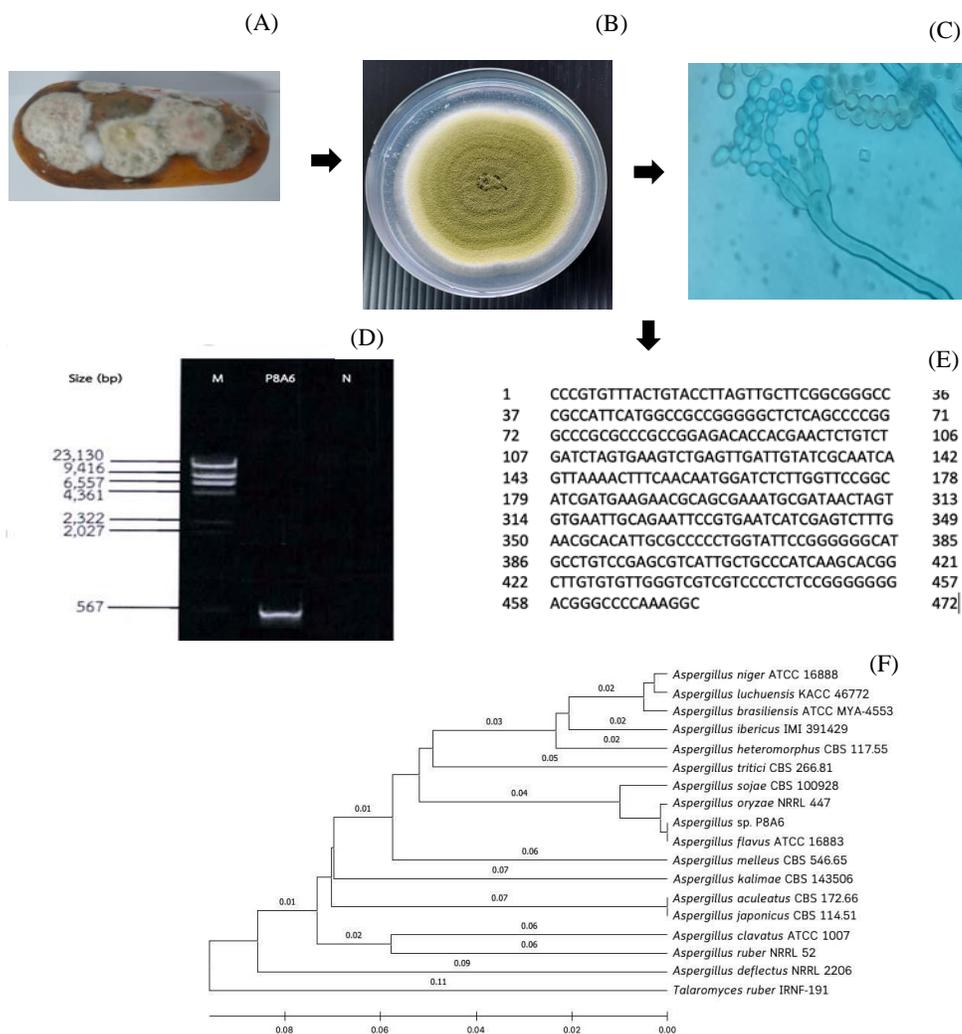
Fig. 2 Morphological and molecular identification of *Aspergillus* sp. P8A6: (A) a moldy papaya; (B) morphology of *Aspergillus* sp. P8A6 on PDA at 30 °C, 7 days; (C) microscopic observation (1,000×); (D) Agarose gel electrophoresis of the PCR products with ITS1–ITS4 target sequences: Lane 1, DNA marker λ/*Hin*dIII; Lane 2, the amplified PCR products; and Lane 3, negative control; (E) Nucleotide sequences of ITS1–ITS4 fragment of PCR products; and (F) phylogenetic tree analysis based on ITS1–ITS4 using Maximum Likelihood method with 1,000 bootstraps.

8 days of storage, papaya treated with edible coating added with 0.0397% clove oil and 0.25% potassium sorbate (T4) displayed the highest percentage of DCE with approximately 50% DCE ($p < 0.05$). The inhibitory effect of ChiCMC against *Talaromyces* sp. P2D4 was not found. However, higher concentrations of chitosan and CMC in the edible coating were previously tested and found that 0.1% chitosan mixed with 0.25% CMC provided the best gel appearance as compared to the higher concentrations. Thus, the inhibitory effect of edible coating should come from the inhibitory effect of clove or cinnamon oil and potassium sorbate.

Clove or cinnamon oil combined with potassium sorbate displayed some synergistic antifungal activity against *Talaromyces* sp. P2D4 on PDA regardless of essential oils and potassium sorbate concentrations. Although clove or cinnamon oil and potassium sorbate only affected moderate disease control, they displayed higher % DCE on papaya fruits as compared to ChiCMC alone (T2) and ChiCMC plus 7,500 ppm chlorothalonil (T3).

b) Physicochemical parameters of papaya fruits

Weight loss

After 4 days of storage, papaya fruits coated with ChiCMC alone (T2), ChiCMC plus 0.0397% clove oil and 0.25% potassium sorbate (T4), and ChiCMC plus

Table 1 Antifungal activity of plant essential oils, pure compounds, and acid salts.

Sample	MIC			
	Aspergillus sp. P8A6	*Aspergillus flavus* TISTR 3041	*Talaromyces* sp. P2D4	*Penicillium citrinum* TISTR 3437
Essential oil (%)				
Cinnamon oil	0.025	0.0125	0.025	0.0125
Clove oil	0.1	0.05	0.2	0.05
Colla aromatica Roxb. oil	0.8	0.2	0.8	0.2
Pure compound (mole/l)				
Cinnamaldehyde	3.78	3.78	3.78	3.78
Eugenol	12.18	6.09	6.09	6.09
Linalool	6.48	6.48	12.97	6.48
Acid salt				
Ammonium carbonate	0.5	0.5	1	0.5
Potassium metabisulfite	1	1	1	1.5
Potassium sorbate	8	2	0.5	2
Sodium benzoate	1	2	>8	1
Chlorothalonil (ppm)	5,000	5,000	50	3,500

Table 2 Synergistic effect of essential oils and acid salts on inhibition of *Talaromyces* sp. P2D4.

Combination	MIC_a		MIC_c		FIC		FICI	Interpretation of FICI
	MIC_{a1}	MIC_{a2}	MIC_{c1}	MIC_{c2}	FIC_1	FIC_2		
Cinnamon oil (1) + Potassium sorbate (2)								
	0.025	0.5	0.0125	0.125	0.5	0.25	0.75	Partial synergism
Clove oil (1) + Potassium sorbate (2)								
	0.2	0.5	0.05	0.125	0.25	0.25	0.5	Synergism
Cinnamon oil (1) + Ammonium carbonate (2)								
	0.025	1.0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Clove oil (1) + Ammonium carbonate (2)								
	0.2	1.0	0.1	0.5	0.5	0.5	1.0	No effect

MIC_{a1} is MIC of oil (1) alone, and MIC_{a2} is MIC of acid salt (2) alone. MIC_{c1} is MIC of oil (1) combined with acid salt (2), and MIC_{c2} is MIC of acid salt (2) combined with oil (1). FIC_1 was calculated by using MIC_{c1} divided by MIC_{a1}. FIC_2 was calculated by using MIC_{c2} divided by MIC_{a2}. Sum of FIC_1 and FIC_2 is FIC index (FICI); FICI of less than 0.5 or equal to 0.5 indicated total synergism (FIC ≤ 0.5); FICI of more than 0.5 but equal or less than 0.75 indicated partial synergism (0.5 < FIC ≤ 0.75); FICI of more than 0.75 but equal or less than 2 indicated no effect (0.75 < FIC ≤ 2); FICI of more than 2 indicated antagonism (FIC > 2); and N.D., not detected.

0.0397% cinnamon oil and 0.25% potassium sorbate (T5) had lower weight loss as compared to the control treatment (T1) (Fig. 3B). However, the papaya fruits coated with ChiCMC plus 7,500 ppm chlorothalonil (T3) and those coated with ChiCMC incorporated with 0.25% cinnamon oil and 1% potassium sorbate (T6) showed significantly higher weight loss (11.80 and 11.99%, respectively) than other treatments at the end of storage ($p < 0.05$).

Total soluble solid, titratable acidity, pH, and mature index

At the beginning of storage, the TSS, pH value, and MI of all papaya fruits treated with *Talaromyces* sp. P2D4 were in the range of 4.40–9.65° Brix, 5.66–5.75 pH value, and 26.32–71.52 MI, respectively. As storage time increased, the TSS, pH value, and MI of most treatments decreased to 6.25–8.05° Brix, 4.41–5.28 pH value, and 28.09–46.09 MI, respectively, at the day 8th of storage time. However, the TA increased as storage time increased, and it ranged from 0.173 to 0.263% at the end of storage time. The samples with 0.25% cinnamon oil and 1% potassium sorbate had the highest MI (46.09) (Table 3).

Significant difference between the TSS of the control treatment (T1) and ChiCMC treatment (T2) at the day 8th of storage ($p < 0.05$) was found. The papaya fruits coated with ChiCMC plus 0.0397% clove oil and 0.25% potassium sorbate (T4) showed significantly lower pH value (4.41) after 8 days of storage than other treatments ($p < 0.05$), while the samples coated

Table 3 Total soluble solid, total acidity, pH value, and mature index of papaya fruits inoculated with *Talaromyces* sp. P2D4 during storage at 30 °C.

Treatment	Analysed value[1] ± SD		
	0 day	4 day	8 day
Total soluble solid (TSS, °Brix)			
T1, Control: sterile water	8.50 ± 0.00^b	8.10 ± 0.57^a	7.60 ± 0.57^{bc}
T2, ChiCMC[2]	4.40 ± 0.00^a	7.45 ± 1.48^a	6.25 ± 0.35^a
T3, ChiCMC + 7500 ppm CTN[3]	8.50 ± 0.00^b	8.50 ± 0.00^a	8.05 ± 0.07^c
T4, ChiCMC + 0.0397% clo[4] + 0.25%PS[5]	9.30 ± 1.13^b	7.45 ± 1.48^a	6.80 ± 0.85^{ab}
T5, ChiCMC + 0.0397% cin[6] + 0.25%PS	9.65 ± 0.64^b	8.10 ± 0.00^a	7.60 ± 0.28^{bc}
T6, ChiCMC + 0.25% cin + 1%PS	8.90 ± 0.57^b	8.50 ± 0.00^a	7.95 ± 0.07^{bc}
Titratable acidity (TA, %)			
T1, Control: sterile water	0.122 ± 0.03^a	0.128 ± 0.00^a	0.179 ± 0.00^a
T2, ChiCMC	0.166 ± 0.00^b	0.141 ± 0.04^a	0.231 ± 0.05^{ab}
T3, ChiCMC + 7500 ppm chlorothalonil	0.148 ± 0.01^{ab}	0.186 ± 0.10^a	0.263 ± 0.03^b
T4, ChiCMC + 0.0397% clo + 0.25%PS	0.128 ± 0.02^a	0.186 ± 0.001^a	0.231 ± 0.02^{ab}
T5, ChiCMC + 0.0397% cin + 0.25%PS	0.166 ± 0.00^b	0.141 ± 0.02^a	0.199 ± 0.01^{ab}
T6, ChiCMC + 0.25% cin + 1%PS	0.179 ± 0.00^b	0.166 ± 0.00^a	0.173 ± 0.01^a
pH			
T1, Control: sterile water	5.73 ± 0.04^a	4.96 ± 0.38^{abc}	5.12 ± 0.02^b
T2, ChiCMC	5.68 ± 0.13^a	5.47 ± 0.06^{bc}	5.19 ± 0.03^{bc}
T3, ChiCMC + 7500 ppm chlorothalonil	5.66 ± 0.06^a	4.84 ± 0.39^{ab}	5.08 ± 0.04^b
T4, ChiCMC + 0.0397% clo + 0.25%PS	5.75 ± 0.05^a	4.40 ± 0.25^a	4.41 ± 0.11^a
T5, ChiCMC + 0.0397% cin + 0.25%PS	5.72 ± 0.13^a	5.43 ± 0.14^{bc}	5.17 ± 0.03^{bc}
T6, ChiCMC + 0.25% cin + 1%PS	5.68 ± 0.22^a	5.53 ± 0.00^c	5.28 ± 0.03^c
Mature index (MI)			
T1, Control: sterile water	71.52 ± 15.97^c	63.05 ± 4.53^a	42.44 ± 3.19^c
T2, ChiCMC	26.32 ± 0.00^a	55.20 ± 23.42^a	28.09 ± 8.15^a
T3, ChiCMC + 7500 ppm chlorothalonil	57.72 ± 3.55^{bc}	53.37 ± 28.62^a	30.83 ± 2.92^{ab}
T4, ChiCMC + 0.0397% clo + 0.25%PS	66.92 ± 9.46^{bc}	45.75 ± 2.23^a	29.46 ± 1.37^{ab}
T5, ChiCMC + 0.0397% cin + 0.25%PS	58.06 ± 3.91^{bc}	57.79 ± 7.43^a	38.31 ± 0.32^{bc}
T6, ChiCMC + 0.25% cin + 1%PS	49.61 ± 3.24^b	51.08 ± 0.17^a	46.09 ± 1.97^c

[1] Data are mean of 3 replications; [2] ChiCMC, 0.1% chitosan mixed with 0.25% carboxymethyl cellulose; [3] CTN, Chlorothalonil; [4] clo, clove oil; [5] PS, potassium sorbate; and [6] cin, cinnamon oil. [a,b,c] Different letters in different rows of the same column indicate significant difference ($p < 0.05$).

with ChiCMC incorporated with 0.25% cinnamon oil and 1% potassium sorbate (T6) showed the lowest TA (0.173%) (Table 3).
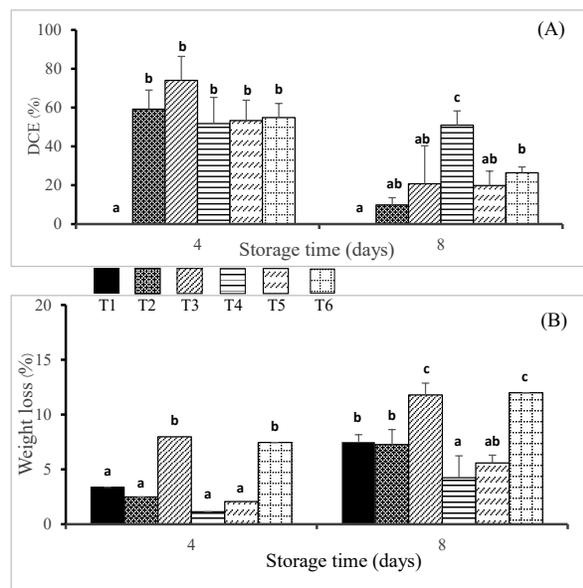
Effect of ChiCMC coating incorporated with essential oils and potassium sorbate on membrane integrity of *Talaromyces* sp. P2D4

Study of edible coating effect on membrane integrity of *Talaromyces* sp. P2D4 indicated that ChiCMC plus 7,500 ppm chlorothalonil (T3 edible coating) affected more morphological damage of *Talaromyces* sp. P2D4 mycelia than other treatments. Many mycelium fragments and morphological alteration were observed as shown in Fig. 4C. Chlorothalonil is a benzonitrile fungicide that is an inhibitor of fungal cell gluthanione molecules, used for black spot, anthracnose, and stem-end rot treatment in papaya fruits [1]. By comparison of the control treatment (T1), hyphae treated with ChiCMC edible coating incorporated with clove or cinnamon oil and potassium sorbate (T4–T6, Fig. 4D-

F) appeared darker blue staining which indicated dead of these fungal cells by allowing the entrance of the Evans blue dye.

Effect of ChiCMC incorporated with essential oils and potassium sorbate on disease incidence of non-inoculated papaya fruits

Papaya fruits used in this test were not inoculated with mold. In the control fruits in which edible coating were not treated, the 100% decay incidence (T1) was shown after 8 days of storage at 30 °C. Edible coating with clove or cinnamon oil and potassium sorbate (T4–T6) significantly decreased the percentage of decay incidence of papaya fruits by 53.3–56.7% ($p < 0.05$). These showed better protection as compared to ChiCMC coating (T2) and ChiCMC with 7,500 ppm chlorothalonil (T3) (76.7% and 83.3% decay incidence, respectively, Fig. 5).

Fig. 3 Effect of ChiCMC incorporated with cinnamon or clove oil combined with potassium sorbate on disease control efficiency, DCE (A) and weight loss (B) in papaya fruits inoculated with *Talaromyces* sp. P2D4. T1, (control (sterile water)); T2, (ChiCMC (0.1% chitosan + 0.25% CMC)); T3, (ChiCMC + 7500 ppm chlorothalonil); T4, (ChiCMC + 0.0397% clove oil + 0.25% potassium sorbate (PS)); T5, (ChiCMC + 0.0397% cinnamon oil + 0.25% PS); and T6, (ChiCMC + 0.25% cinnamon oil + 1% PS).

DISCUSSION

In the current study, most of spoiled ripe papaya fruits were highly contaminated with fungi. Several mold strains were isolated, but the most interesting one was *Talaromyces* sp. P2D4. *Talaromyces* is a sexual state of *Penicillium* containing many species which belong to the Order Eurotiales. Some species of *Talaromyces* are food-spoiling fungi which cause postharvest devastation of food plants [24]. *Talaromyces* sp. P2D4 produced orange red pigment. According to Yilmaz et al [25], *Talaromyces* normally produces orange and red or weak red pigments as soluble pigments. Some species of *Talaromyces* produce rugulosin, a bis-anthraquinoid pigment. Thus, *Talaromyces* species are considered an important fungus for biotechnological purposes.

Cinnamon and clove oils possessed strong antifungal activity which was in agreement with those reported by Ju et al [26]. Cinnamon and clove oils could inhibit the growth of *Penicillium* spp. and *Aspergillus* spp. at 0.21–0.83 and 0.21–1.67 µg/ml, respectively, demonstrating the stronger antifungal activity of cinnamon oil than clove oil [26]. This was probably due to their active constituents. Huang et al [27] identified active compounds in cinnamon stem bark oil by gas chromatography-mass spectrometry (GC-MS)

and found that the main compounds in cinnamon oil were cinnamaldehyde (85.78%) and diethyl malonate (7.3%). Similarly, clove oil has been reported to contain several active compounds, especially its main compounds including eugenol up to 90%, eugenyl acetate, *p*-cymene, β-caryophyllene, and *trans-p*-2-menthen-1-ol [28]. However, Aiemsaard et al [29] reported that the main components of clove oil were eugenol (98.87%) and trans-caryophyllene (1.13%). Colla aromatica Roxb. oil showed relatively strong antifungal activity. This was probably due to the action of its active compounds. Active compounds in colla aromatica Roxb. oil were reported to be linalool in large amounts and other compounds such as terpene-4-ol, δ-cadinene, α-cadinol, and others in small amounts [4]. The higher concentration of cinnamon or clove oil and ammonium carbonate or potassium metabisulfite increased mycelial growth inhibition. Cinnamon oil contains cinnamaldehyde and eugenol, while clove oil has eugenol as the main constituent which contains more phenolic compounds in its structures [26, 27]. Wangprasertkul et al [30] revealed antifungal activity of ammonium carbonate, potassium sorbate, sodium metabisulfite, and sodium benzoate against *Aspergillus*, *Fusarium*, and *Rhizopus*. This could affect their strongest antifungal activities. These compounds can damage fungal cell wall and finally result in cell death [26].

The use of chitosan to control fungal disease in papaya fruits has been reported [31], while CMC in combination with essential oils has been reported to extend the shelf life of papaya fruits [32]. Previous studies have shown that clove or cinnamon oil combined with chitosan displayed postharvest fungal control in fruits. The mechanism of action of clove or cinnamon oil may relate to the action of their active compounds. Essential oils possess active components with great hydrophobicity, allowing them to partition lipid bilayers of fungal cytoplasmic membrane. These result in disruption of membrane structure, alteration of ion gradients, and modification of cell pH, finally affecting cell metabolic processes and ultimately leading to cell death [33].

The physiological loss of weight was probably due to the respiration of the fruit. The respiratory degradation process of plant reserving carbohydrate produces carbon dioxide and water as end products, resulting in transpiration of water through their peel. In some treatments, the use of chitosan and CMC coating may help to decrease oxygen, carbon dioxide, and moisture, thereby decreasing fruit respiration rate and weight loss [34]. Edible coating containing various active ingredients could decrease microbial growth and weight loss and prevent escape of some volatile compounds. Active components released from the edible coating onto fresh papaya fruits could help to protect them from undesirable reactions such as
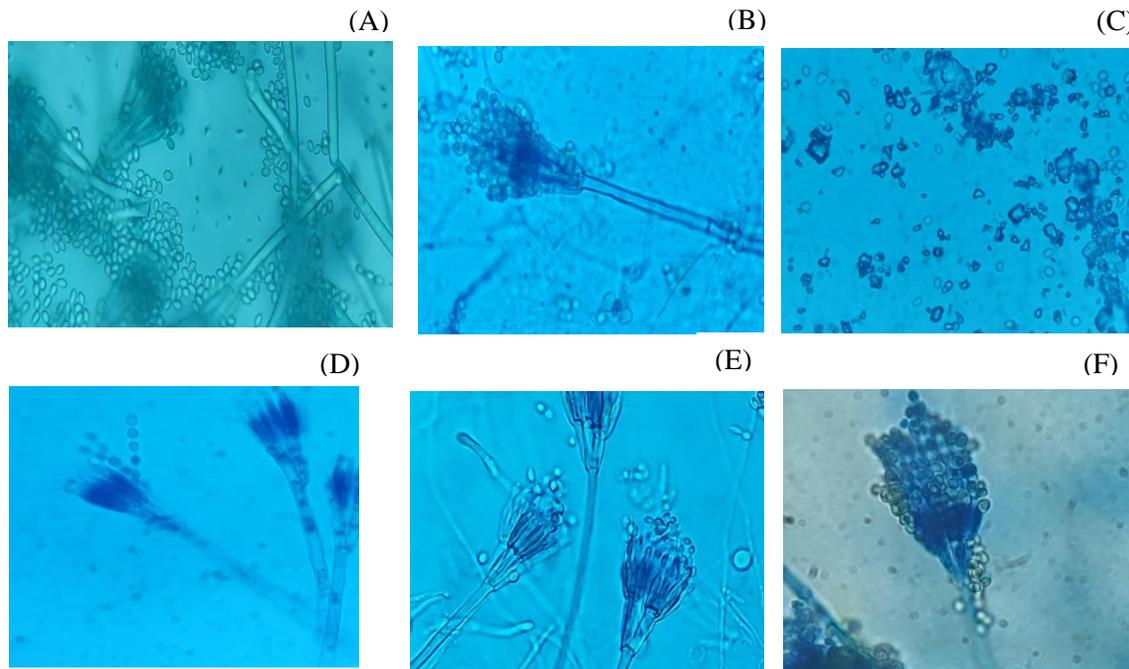
Fig. 4 Microscopic observation (400 × total magnification) of *Talaromyces* sp. P2D4 after treatment with edible coating. (A) T1 (Control (sterile water)); (B) T2 (ChiCMC (0.1% Chitosan + 0.25% CMC)); (C) T3 (ChiCMC + 7500 ppm chlorothalonil (CTN)); (D) T4 (ChiCMC + 0.0397% clove oil (Clo) + 0.25% potassium sorbate (PS)); (E) T5 (ChiCMC + 0.0397% cinnamon oil (Cin) + 0.25% PS); and (F) T6 (ChiCMC + 0.25% cinnamon oil + 1% PS).
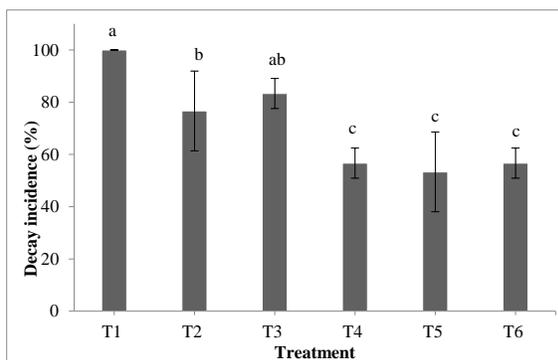
Fig. 5 Effect of ChiCMC incorporated with essential oils and potassium sorbate (PS) on disease incidence of non-inoculated papaya fruits after 8-day storage at 30 °C: T1 (Control, Sterile water); T2 (ChiCMC, 0.1% Chitosan + 0.25% CMC); T3 (ChiCMC + 7500 ppm chlorothalonil); T4 (ChiCMC + 0.0397% clove oil + 0.25% PS); T5 (ChiCMC +0.0397% cinnamon oil + 0.25% PS; and T6 (ChiCMC + 0.25% cinnamon oil + 1% PS).

ethylene production, discoloration as well as bacterial and fungal growth [6]. In addition, titratable acidity of papaya pulp increased while pH value decreased regardless of coating applications. This indicated the presence of acids in papaya fruit pulps. These were in agreement with the study reported by Mendy et al [35]. Dotto and Abihudi [36] reported that 100 g papaya fruits contained 35.32–43.80 mg ascorbic acid and 17.76–13.44 mg carbohydrate. Moreover, papaya fruits also contain phytochemicals such as carotenoids, β-carotene, cryptoxanthin, lycopene, violaxanthin, zeaxanthin, and flavonoids including kaempferol, myricetin, and quercetin [37].

CONCLUSION

This study confirmed the antifungal effect of clove, cinnamon, and colla aromatica Roxb. oils, and some acid salts and synergistic effect of clove or cinnamon oil with potassium sorbate. The incorporation of clove or cinnamon oil at 0.0397–0.25% concentration plus 0.25–1.0% potassium sorbate into ChiCMC edible coating could help to extend the shelf life of papaya fruits by at least 50%.

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