CHAPTER 5

EFFECTS OF COATING COMPONENTS ON DISEASE SEVERITY, FUNGAL GROWTH AND MODE OF REACTION ON *LASIODIPLODIA THEOBROMAE*

5.1 Abstract

The effect of chitosan and its components: citric acid (CA) and potassium sorbate (PS) on the growth and morphology of *Lasiodiplodia theobromae*, one of the major postharvest pathogens of fresh longan fruit were investigated *in vitro* and *in vivo*. The results of inhibition of spore germination and radial growth of *L. theobromae in vitro* indicated that three components could efficiently inhibit the growth of the tested fungi. The effectiveness of coating components [0.3% PS, 3.0% CA and 1.2% Cts] to control postharvest decay and the mode of reaction of the components against this fungus on longan fruits stored at 18˚C and 25˚C was also investigated. Cts and PS, CA treatments, or combined, significantly reduced postharvest fungal rot of the fruit compared with controls challenged with *L. theobromae*. 1.2% Chitosan+0.3% PS+3.0% CA combination showed high efficacy to delay the lowest disease development compared to those treated with 0.3% PS+3.0% CA and 0.3% PS. In regards to Cts component on pathogenesis-related protein (PR-protein): chitinase; and ß-1, 3-glucanase activity. It was found that PR-proteins were slightly induced but not sufficient to delay disease development. The enzyme induction was mostly related to wounding and pathogenic infection. Chitosan along with PS+CA maintained the highest amount of sorbic acid content in the pericarp, which is known to be of great importance in longan resistance to this fungus. Chitosan could be used to a carrier of PS with CA as a stabilizer to protect longan fruit against postharvest disease

5.2 Introduction

Fresh 'Daw' longan fruit is the leading product to export in Thailand. It is considered a short shelf life commodity, due to a rapid discoloration process and also highly susceptible to various postharvest pathogens. The most important pathogens of longan fruit is *Lasiodiplodia theobromae* which cause stem-end rot disease (Suwanakood *et al*., 2007). It was found as a latent infection and an endophytic fungus in longan which was founded on the many parts of longan tree (Sardsud *et al*., 1998). To overcome this problem, sulfur dioxide (SO_2) fumigation are applied for controlling this disease (Tongdee, 1994). However, use of SO_2 has resulted in serious problems of SO_2 residues in the food product and there have been numerous reports on toxic residues in humans as well as reactions in sensitive individuals and asthmatics (Rank and Li, 2007). Thus, alternative control strategy has been stimulated to do the research. Recently, chitosan coating interested to apply for reducing not only disease incidence in longan (Jiang and Li, 2001) and litchi (Zhang and Quantick, 1997) but it also maintained high fruit quality.

Apai *et al*. (2008a) showed that chitosan mixed with citric acid could control browning in longan fruit when compared to use citric acid alone, but some diseases can attack and short shelf-life was occurred. However, antimicrobial of chitosan coating can be improved because it has been reported a good properties such as biocompatibility and carriers with many food additives, antioxidant, mineral, antagonist etc., (Cuppett, 1994) for improving its high efficacy which depended on their objective uses. Baldwin *et al*. (1996) reported that potassium sorbate incorporated with edible coating in fresh-cut apple and potatoes reduced volume of mold and yeast lower than those treated with potassium sorbate alone. Park *et al*. (2005) showed significant different *in vitro* study on mycelia growth of *Cladiosporium* sp. and *Rhizopus stolonifer* between potassium sorbate mixed in PDA and chitosan, but *in vivo* lab was contrasted. Preliminary study showed that dipping longan fruit in 0.3% potassium sorbate (PS)+1.2% chitosan (dissolved in 3.0% citric acid) showed lower disease incidence (%) and better pericarp skin color than those dipped in PS alone (data not shown) and it was selected to investigated the antifungal activity of the coating and component. This study was to evaluate the antifungal

activity of this treatment on the important fungi: *Lasiodiplodia theobromea in vitro* and *vivo* study.

5.3 Materials and methods

5.3.1 Plant material

Mature longan cv Daw were harvested and selected for uniformity, shape, colour, and size and any blemished or diseased fruits were discarded. They were used to investigate antifungal activity *in vivo* experiment.

5.3.2 Fungal culture and growth condition

Lasiodiplodia. theobromae LP20, virulent isolate, (which cause the most severe symptoms and rot the experimental longan fruits rapidly) provided by Suwanakood (2007). 0.5 cm mycelia disks culture was placed in Petri dishes containing potato dextrose agar and incubated at ambient temperature (25°C) for 3 days before use. *L. theobromae* conidium was produced on fresh mangosteen fruit. The fruits were inoculated on artificially wounded areas with the mycelial disks of *L. theobromae* and incubated in a moist chamber (98–100% RH.) at 25°C for 2 days. The inoculated fruit was further incubated at the ambient temperature for 3 weeks. Conidia of *L. theobromae* produced on the fruit surface were brushed off, collected in a jar, and then dried in silica gel. These conidia were used for experiments which follow (Sangchote and Saoha, 1998).

5.3.3 Antifungal assays (*in vitro* trials)

5.3.3.1 Antifungal activity of coating component on the spore germination and the mycelium growth

Poisoned food technique was used. 3.0% (w/v) chitosan from flake shrimp (high molecular weight, 93.75%DD) was dissolved in hot citric acid solution at a concentration of 3.0% (w/v). The PDA was supplemented with chitosan at the final concentrations of 0, 0.06, 0.12, 0.6, 1.2 and 1.5% (v/v). Stock solution of food grade citric acid (CA) and food grade PS were individually mixed with PDA and final concentrations were: 0, 0.3, 0.6, 1.0, 3.0 and 6.0% (v/v) for CA and 0, 0.03, 0.06, 0.1,

0.3 and 0.6% (v/v) for PS.

The efficacy of PS with or without combination with chitosan coating was also investigated. PS solution was incorporated into plain PDA or PDA with 1.2% chitosan, each of which contained 3.0% CA and final PS concentrations were: 0, 0.03, 0.06, 0.1, 0.3 and 0.6% (v/v). The change in pH of PDA medium after mixed with coating component were measured using a digital pH meter (Consort C831, Belgium).

Two methods were used to determine the effect of coating components on the growths of *L. theobromae* LP20. In the first method, a spore suspension was obtained by mixing of conidia *L. theobromae* LP20 with sterile distilled water containing 0.1% (v:v) Tween 80 and filtered through cheesecloth to remove most of the mycelium fragments and then adjusted to a concentration of 10^6 conidia/ml. Spore concentration of the pathogens was measured using a haemacytometer. For each treatment, five replicate plates were used. They were incubated at ambient temperature (25°C) for 6 hours and then, the number of germinated spores per plate was counted. Data were expressed as the percentage of germinated spore's inhibition.

In the second method, 0.5 cm mycelial disk of *L. theobromae* LP20 was placed at the center of the test medium. There were five (plate) replications per treatment and the plates were incubated at ambient temperature (25°C) for 3 days. For each treatment, five replicate plates were used. In addition, after 3 days of incubation at 25°C, the inhibition of the mycelial growth was observed after the mold in the control plate had grown to the edge of the plate. Radial growth reduction was calculated as follows: (a – b) x 100 / a (where a = radial growth measurement of the pathogen in the control and b is that of the pathogen in the presence of the coating component tested); these values were then expressed as inhibition percentage of radial mycelial growth. The nature of the antifungal activity, fungistatic or fungicidal, was determined by the method of Garber and Houston (1959). After 3 days, the fungal mycelial disks which were unable to grow were transferred onto fresh PDA medium and incubated further for 7 days.

5.3.3.2 Light microscopic and scanning electron microscope (SEM) of morphemically changes on fungal morphology

To further characterize the antifungal activity, we carried out a parallel study using PDA plates containing coating components as mentioned above. The component concentration from mycelium growth test that exhibited fungal activity was subjected to further cell morphological characterization which was observed under a light microscope following the method of Ait Barka *et al*. (2004). A thin layer of PDA was aseptically removed and placed in a drop of sterile water on a glass slide. A cover slip was placed on the top the suspension and observation was made with the light microscope (Olympus, Japan).

The morphological changes of fungal cell as affected by coating component were emphasized using SEM. Effects of coating components on morphemically change was elucidated by scanning electron microscope (SEM). To study the morphological change of mycelium PDA that supplement with coating components, all peel specimens (1 mm thick), cut in pieces of 5x5 mm^2 from the ridge of PDA plates after 3 days, were prepared for scanning electron microscopy (SEM, JEOL JSM-5910LV). The hypha specimens of the pericarp were being immersed into chilled 2.5 % glutaradehyde in 0.1 M phosphate buffer pH 7.2 washed with 0.1 M phosphate buffer pH 7.2. Dehydrated the hypha specimens through the series 30%, 50%, 70%, 80%, 90%, 95%, 100%, 100% acetone, allowing 30 min in each before being critical-point dried. The prepared hypha specimens were mounted on copper stubs and sputter coated with gold. The tissue specimens were viewed with the scanning electron microscope.

5.3.4 Efficacy of integrated methods for controlling fruit rots development

5.3.4.1 Disease development, SEM of infection site and sorbic acid in pericarp

The effects of 1.2% (w/v) Cts, 3.0% CA and 0.3% PS and their combinations on fruit rot development were evaluated in two different experiments. For the first experiments, the sorbic acid in coating components was studied. The longan fruits

were artificially inoculated. A 0.5 cm diameter of *L. theobromae* LP20 mycelial disk was inoculated on the top of artificial wounds made by nipping the stalk of the stem-end area of each fruit and incubated in a high moisture box at room temperature for 6 hours (Figure 5.1).

Figure 5.1 A 0.5 cm diameter of *L. theobromae* LP20 mycelial disk was inoculated on the top of artificial wounds made by nipping the stalk of the stem-end area of each fruit and incubated in a high moisture box at room temperature for 6 hours.

For the first experiment, the inoculated fruits were then dipped for 5 min in the 0.3% PS with 1.2% chitosan (dissolved in 3.0% CA) and their components: 0.3%PS+3.0%CA, 0.3%PS and 3.0%CA. The treated fruits were air dried and kept in foam trays, wrapped with 11 μm PVC. Each treatment comprised three replicates. In the first experiments, all treated fruit were stored at 25ºC for 72 hrs. The fruits were evaluated disease severity of inoculated fruit, SEM on inoculation site and sorbic acid degradation in pericarp.

5.3.4.2 Mode of reactions of coating component on disease severity and PR-protein as a defense mechanism

For the second experiment, the natural defense enzyme (chitinase and β-1, 3-gulcanase activity) on disease development was evaluated. For the second experiments, the fruit with or without inoculation were used. They were then dipped for 5 min in the 0.3% PS with 1.2% chitosan (dissolved in 3.0% CA) (pH 2.8 and 3.3) and its component: 0.3%PS+3.0%CA and control fruits (non-treated fruit). Each treatment comprised three replicates. The treated fruit were stored at 20ºC for 5 days. Disease severity of the inoculated fruits and disease incidence percentage for non-inoculated fruit and defense enzymes (chitinase and β-1, 3-gulcanase activity) were analyzed.

-Disease severity

Disease severity was measured at different time during storage and expressed as the scores of disease severity; 1= no symptom, 2 = 1-10% infection of stem end area, 3=10-25%, 4=26-50%, 5= more than 50% infection of fruit area.

- Sorbic acid degradation

Sorbic acid (SA) degradation in the part of fruit pericarp and flesh were analyzed by modifying method of Kamlert (1992). Five fruit in each of the three replications were used to prepare sorbic acid samples. Pericarp and flesh were removed from the fruit at the time of decaying analysis, finely ground using the Moulinex blender and weighing about ~5.00 g in 100 ml volumetric flask. Adding 80 mL of extract solution (HPLC grade methanol : 0.01 M ammonium acetate buffer (pH 4.5-6) (60:40) and each of 1 mL of Carrez I (15% potassium ferro cyanate) and Carrez II (23% zinc acetate) respectively was shaken by hand thoroughly, adjusted volume to 100 mL and stored at 25 °C for 15 min. The solutions were filtered through no. 42 paper filters and then through a 0.45-µm nylon membrane filter before injection. The sorbic acid concentration in each solution was determined using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with a diode array detector, auto sampler, Hypersil BDS C18 column (inner dia, 150 × 5 mm) with guard column and computer with chemstation software. The mobile phase was methanol: 0.01 M

ammonium acetate buffer (pH 4.5-4.6) (60:40), the injection volume was 20 µL and the flow rate was 1.0 mL/min. Absorbance was read at 235 nm and run time was 10 min. Calibration curve was created by diluting PS calculation as sorbic acid in the concentration ranges from 0.01 to 100 mg/L. The method recovery test as the accuracy of sorbic acid analysis was done by spiking 20 mg/L SA (middle range) in the blank samples and it was 93-96%. The determination of linearity (R^2) of the standard curve was 0.9995. The SA contents in pericarp or flesh were expressed as mg/kg.

- SEM of *L. theobromae* on infection site

To study the morphological change of mycelium on inoculated site, all peel specimens (1 mm thick), cut in pieces of 5x5 mm^2 from inoculated site on stem-end fruits after 24 and 48 hrs of inoculation, were prepared for scanning electron microscopy (SEM, JEOL JSM-5910LV). Tissue specimens of the pericarp were being immersed into chilled 2.5 % glutaradehyde in 0.1 M phosphate buffer pH 7.2 washed with 0.1 M phosphate buffer pH 7.2. Dehydrated the tissue specimens through the series 30%, 50%, 70%, 80%, 90%, 95%, 100%, 100% acetone, allowing 30 min in each before being critical-point dried. The prepared tissue specimens were mounted on copper stubs and sputter coated with gold. The tissue specimens were viewed with the scanning electron microscope.

-Pathogenesis related- protein as a defense mechanism

In preparing the crude chitinase and ß-1, 3-Glucanase, the peels from five fruits of each replication were frozen with liquid nitrogen and then powdered using the Moulinex blender. Frozen tissues (3.0 g) were homogenized in 9 ml of 50 mM sodium acetate buffer, pH 5.0 at 4°C. The homogenate was centrifuged at 4,100 x g for 20 min and then supernatant was collected to assay.

Chitinase activity was assayed using swollen chitin (Monreal and Reese, 1969) following the method of Reissig *et al*. (1955), with slight modifications. Chitinase activity was measured by mixing 1 ml of crude enzyme solution with 1 ml of 1% swollen chitin in 50 mM citrate buffer (pH 6.6). After incubation at 50˚C for 60 min, the reaction was stopped by boiling for 5 min. 0.5 ml supernatant and 0.1 ml

of 0.8 M $K_2B_4O_7$ were mixed and boiled for another 3 min, then placed in an ice bath and 3 ml distilled DMAB was added and incubated for 20 min at 37˚C. The optical absorption was measured at 585 nm using a UV/Visible Spectrophotometer (PerkinElmer lambda 35, USA). The amount of N-acetylglucosamine (NAG) released was calculated from a standard curve prepared with NAG and the chitinase activity was expressed in units (U/mg protein).

ß-1, 3-Glucanase activity was determined by a colorimetric method (Burner, 1964). The amount of reducing sugar released from laminarin was measured. The standard assay contained 50 µl of the crude enzyme solution and 50 µl of 5 mg/ml laminarin in 0.1M sodium acetate buffer pH 5.0. After incubation at 35˚C for 30 min, the reaction was stopped by boiling for 5 min and 0.2 ml of 1% dinitrosalicylate (DNS) and 0.2 ml of sodium acetate buffer were added and boiled for another 5 min, then placed in an ice bath and 0.9 ml sterile distilled H_2O was added. The optical absorption was measured at 540 nm. The amount of reducing sugar released was calculated from a standard curve prepared with glucose and the glucanase activity was expressed in units (U/mg protein). The protein content was determined according to the dye-binding method of Bradford (1976) using bovine serum albumin as the standard.

5.3.5 Statistical Analysis

Analysis of variance (ANOVA) and the test of mean comparison according to least significant difference (LSD) were applied with a significance level of 0.05. The SPSS software version 10 for Windows was used as a statistical analysis tool.

5.4 Results and discussion

5.4.1 Antifungal activity on *in vitro* study

5.4.1.1 Antifungal activity of coating component on the spore germination and mycelium growth

The *in vitro* experiment showed that chitosan acidified by CA increased its inhibiting efficacy to spore germination and hyphal growth as the concentration increased. 0.6-1.2% (v/v) chitosan provided high inhibiting efficacy on mycelium growth at 89.88-92.24%. 0.12-1.2% (v/v) provided high inhibiting efficacy on spore germination at 91.17-100%. This indicated that the growth inhibition increased as medium pH decreased which caused by the increasing in CA (%v/v) in chitosan (Table 5.1). In another study, chitosan at different concentrations (0.01–1%) markedly inhibited mycelial growth of *Botrytis cinerea* and *Penicillium expansum* (Liu *et al*., 2007) and *L. theobromae* (Jitareerat *et al*., 2008). It is generally assumed that the cationic nature of chitosan (pKa 6.3), conveyed by the positively charged NH_3 groups of glucosamine, might be a fundamental factor contributing to its interaction with the negatively charged microbial cell surface. It was suggested that the cationic amine group (NH_2) increased ionization as pH decreased and could react with anionic ion in the cell membrane causing leakage (Leuba and St¨ossel, 1986; Liu *et al*., 2007). On the other hand, it is claimed that some of chitosan's characteristics, such as its water-binding capacity as well as its abilities to chelate trace metals and to interact with DNA, might shed some light on its antimicrobial mode of action (Rabea *et al*., 2003).

The solution of PS and CA at the concentration of 0.3% and 3.0% completely inhibited the mycelial growth, however inhibitory action against *L. theobromae* LP20 was only fungistatic (Table 5.1). The results from the fungal mycelial disks showed that *L. theobromae* LP20 was able to grow after transfer onto fresh PDA medium and incubated further for 7 days. Antifungal activity of these preservatives was more fungistatic than fungicidal (Palou *et al*., 2002). The main antimicrobial effect of sorbic acid has been attributed to the undissociated acid penetrating the microbial cell wall and then disassociating in higher pH cytoplasm. The H^+ released was believed to inhibit glycolysis and growth (Stratford and Anslow, 1996).

Table 5.1 Antifungal activity of coating components on the growth of *L. theobromae*.

Components[2]	%	pH	Spore inhibition (%)	Mycelium growth inhibition (%)	Nature[4]
Control	0	6.0	0.00 f	0.00 g[1]	fS
Cts	0.06 (0.06)[3]	4.3	3.79 e	16.94 f	fS
	0.12 (0.12)	3.9	91.71 b	80.94 c	fS
	0.6 (0.60)	3.1	93.84 b	89.88 b	fS
	1.2 (1.20)	2.8	95.02 b	92.24 b	fS
	1.5 (1.50)	2.6	100.00 a	92.71 b	fS
CA	0.3	2.9	5.92 e	18.59 f	fS
	0.6	2.3	37.91 c	61.18 d	fS
	1	2.1	100.00 a	88.47 b	fS
	3	1.7	100.00 a	100.00 a	fS
	6	1.4	100.00 a	100.00 a	fS
PS	0.03	5.1	4.98 e	30.35 e	fS
	0.06	5.6	17.3 d	56.94 d	fS
	0.1	5.8	44.68 c	98.59 a	fS
	0.3	6.1	100 a	100.00 a	fS
	0.6	6.5	100 a	100.00 a	fS
CV.			2.29	5.25	

[1]Same letters in the same column are not significantly different at 0.05.

[2]Components; CA = citric acid, Cts = chitosan, PS = potassium sorbate, control = only *L. theobromae* and non-supplemented PDA with coating component.

[3]Citric acid (%V/V) in chitosan

[4]fS = fungistatic activity (mycelium growth inhibition)

The antimicrobial activity was therefore very dependent on the pH as reported by Sofos and Busta (1981), who found the best activity was due to the undissociated form of the acid when pH was less than pKa (pKa-4.75). Mixing of PS with an acidulant agent such as CA increased the inhibiting efficiency on the hyphal growth. Mixing PS at a concentration of 0.3% in 3.0% CA with or without 1.2% (v/v) chitosan

showed the sign of fungicidal activity (Table 5.2). The results were complied with spore inhibition, the combination of 0.3% PS and 3% CA with or without 1.2% chitosan achieved a significant result, inhibiting the spore growth to 100%. The inhibition was resulted from the combination effects between chitosan and PS in acidic solution. Kenneth and Leonard (2005) reported that 0.075% PS with CA at pH 4.1 could change the activity from fungistatic to fungicidal. PS in CA solution with or without chitosan showed no significance in mycelial growth inhibiting percentage (Table 5.2). This result suggested that combinations of coating components had synergistic effect on mycelial growth inhibition and survival of diseases.

Table 5.2 Antifungal activity of PS concentration with or with out coating component on *L. theobromae in vitro* study

Cts[2]	PS (%)	pH	Spore inhibition	Mycelium growth Inhibition	Nature[4]
3.0% CA	0	1.7	100	100.00a[1]	fS
	0.03	1.8	100	100.00a	fC
	0.06	1.8	100	100.00a	fC
	0.1	1.8	100	100.00a	fC
	0.3	2.1	100	100.00a	fC
	0.6	2.3	100	100.00a	fC
1.2%Cts	0	1.8	100	93.18b	fS
+3.0% CA	0.03	1.9	100	100.00a	fC
	0.06	2.0	100	100.00a	fC
	0.1	2.0	100	100.00a	fC
	0.3	2.2	100	100.00a	fC
	0.6	2.4	100	100.00a	fC
CV.				0.28	

[1]Same letters in the same column are not significantly different at 0.05.

[2]CA = citric acid, Cts = chitosan, PS = potassium sorbate

[3]fS = fungistatic activity, fC = fungicidal acitivity (mycelium growth inhibition)

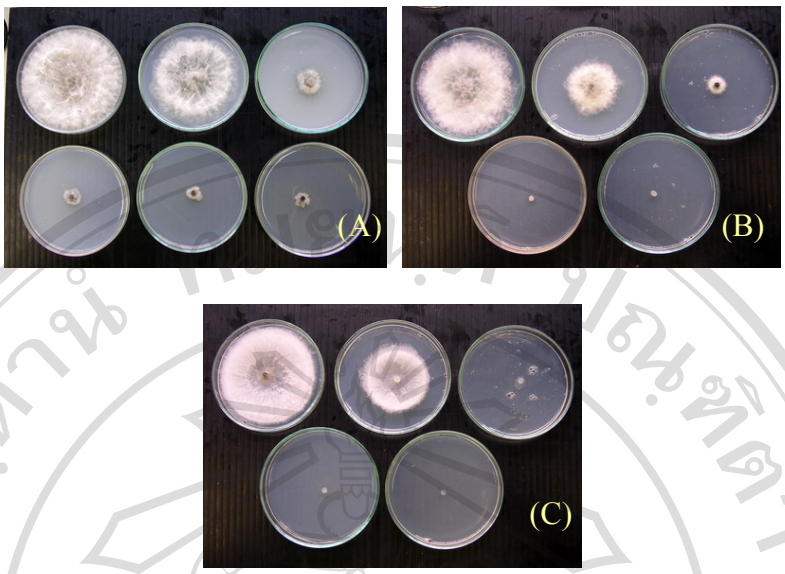
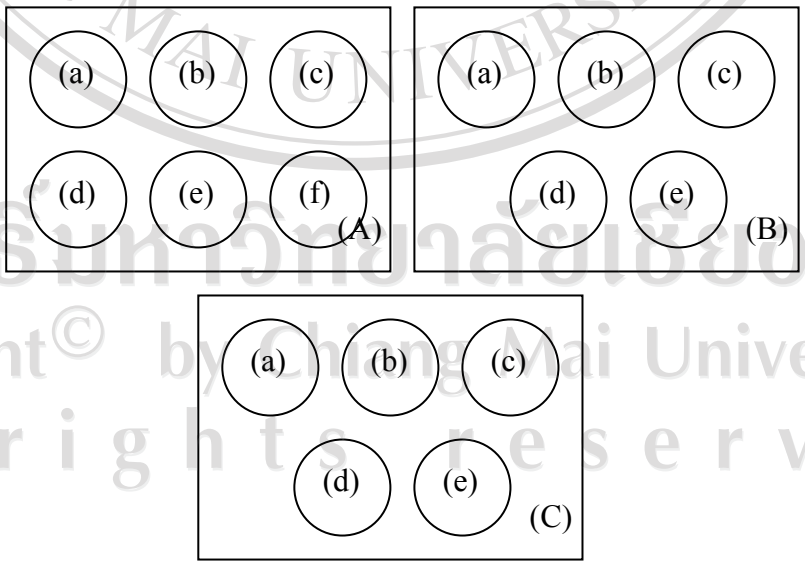


Figure 5.2 Mycelium growth *of L. theobromae* in PDA supplement with chitosan (A), citric acid (B) and potassium sorbate (C).

A; (a) control PDA plate, (b)–(f) chitosan-supplemented PDA plates
: b 0.06, c 0.12, d. 0.60, e 1.2 and f 1.5% (v/v).

B; (a)-(e) citric acid-supplemented PDA plates
: a 0.3%, b 0.6%, c 1.0%, d 3.0%, e 6.0%.

C; (a)-(e) potassium sorbate-supplemented PDA plates
: a 0.03%, b 0.06%, c 0.10%, d 0.30%, e 0.60%.



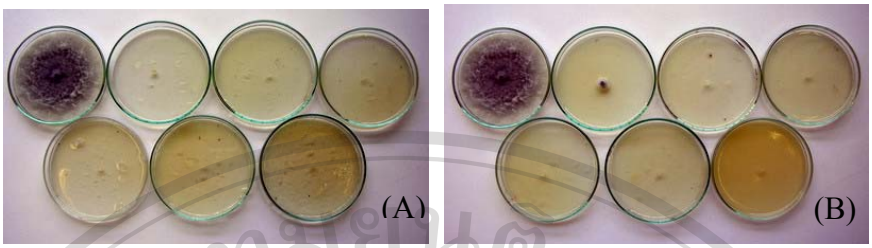
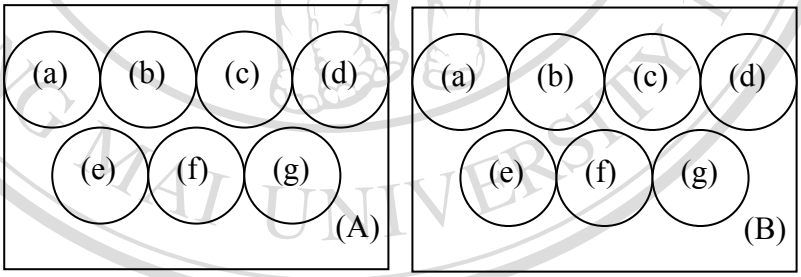


Figure 5.3 Mycelium growth *of L. theobromae* in PDA supplement with PS concentration with or with out coating component

A; various concentration of potassium sorbate+constantly 3.0% citric acid (a) control PDA plates, (b)-(g) potassium sorbate supplemented PDA plates that mixed with constantly 3.0% citric acid: b 0, c 0.03, d 0.06, e 0.10, f 0.30 and g 0.60% (v/v).

B; various concentration of potassium sorbate+constantly 3.0% citric acid+1.2%chitosan (a) control PDA plate, (b)-(g) potassium sorbate supplemented PDA plates that mixed with constantly 3.0% citric acid+1.2%chitosan: b 0, c 0.03, d 0.06, e 0.10, f 0.30 and g 0.60% (v/v).



5.4.1.2 Light microscopic and scanning electron microscope (SEM) of morphemically changes on fungal morphology

- Light microscope

Light microscopic of mycelium at 0.6% chitosan revealed that it could induce abnormal mycelium like swelling and shatter characteristic (Figure 5.4b). Chitosan has been found to affect cell wall composition, morphology, ultrastructure of two wood-inhabiting fungi (Vesentini *et al*., 2007) and also induced morphological changes in *Botrytis cinerea* (Ait Barka *et al*., 2004) and *Rhizopus stolonifer* (El Ghaouth *et al*., 1992).

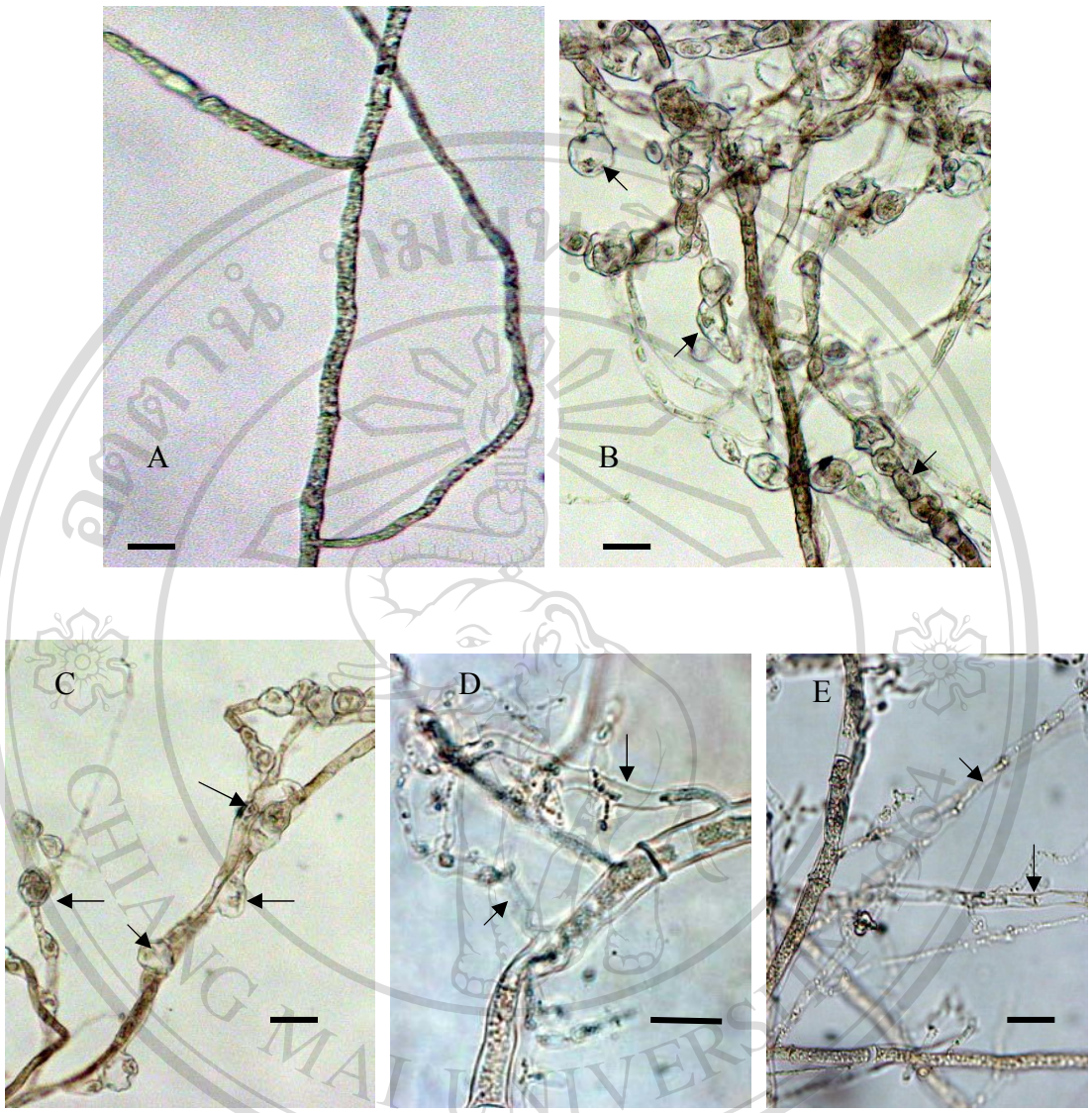


Figure 5.4 Light microscopic structural changes in *L. theobromae* mycelium in response to components in PDA. (A), control (200x), normal hypha; (B) 0.6%Cts (200x), severely damaged hypha were swollen and explosive (C) 1.0%CA (200x), hypha were laterally swollen and (D) (400x) and (E) (200x) 0.10%PS abnormal small branched hypha with empty cytoplasm (arrows).

Hyphae of *L. theobromae* LP20 exposed to 0.6% chitosan showed pronounced vacuolation; sometimes hyphae appeared almost empty. The increase in abnormal characteristic was directly proportional to concentrations of chitosan used, with the highest concentration showing the greatest affect. A change in hyphal diameter and length was also found, the general trend being a swelling in hyphal diameter and length with increasing chitosan concentrations. Chitosan induced swollen mycelium was similar to the effect of 1.0% CA (Figure 5.4c) but swollen mycelium in CA was less severe than those treated with chitosan. Therefore, this swollen mycelium might be affected by components, chitosan and CA. 0.1% PS also induced changes in morphology showing abnormal branches of smaller hypha with clear cytoplasm (Figure 5.4d, e). This result could hypothesize that the H^+ released was believed to inhibit glycolysis and growth (Stratford and Anslow, 1996).

- Scanning electron microscopy (SEM) of *L. theobromae*

The treated with chitosan-treated *L. theobromae* LP20 hyphae provided evidence of seriously morphological changes in hyphal cells at all concentrations of chitosan used, with higher concentrations causing more severe alterations (Figure 5.5). Chitosan also severely affected fungal morphology. Increasing concentrations of chitosan induced excessive branching, vacuolation and a reduction in hyphal diameter (Singha *et al.,* 2008). This suggests that the plasma membrane may be the primary target of chitosan action, and that the fungus differ in the extent to which they are affected. Alterations in fungal morphology can be indicative of compromised plasma membrane, among a host of other cellular changes that are no doubt induced by chitosan. Protein secretion in filamentous fungi occurs around the apical and subapical region of the advancing hyphal tip (Gordon *et al*., 2000; Muller *et al*., 2002). Chitosan was found to react with both the cell wall and the cell membrane, but not simultaneously, indicating that the inactivation of *E. coli* by chitosan occurs via a two-step sequential mechanism: an initial separation of the cell wall from its cell membrane, followed by destruction of the cell membrane (Chung and Chen, 2008).

Control mycelium

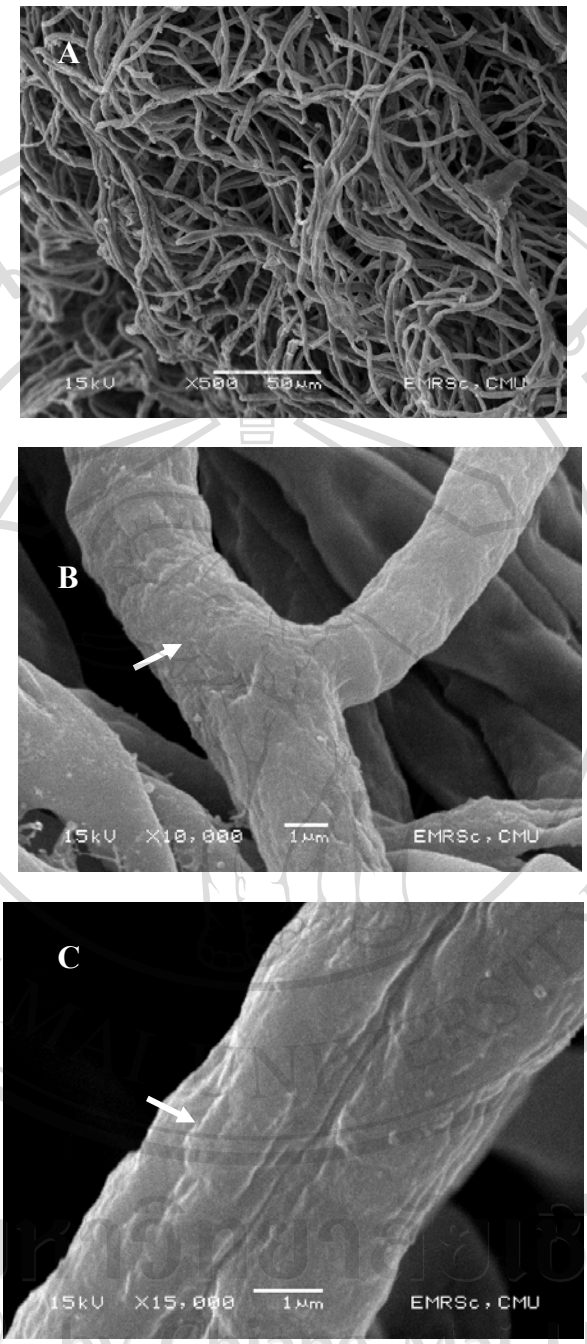


Figure 5.5 Scanning electron micrographs of *L. theobromae* mycelium in control mycelium. (A), control mycelium; (B)-(E) no corrugugated mycelium.

Chitosan mycelium

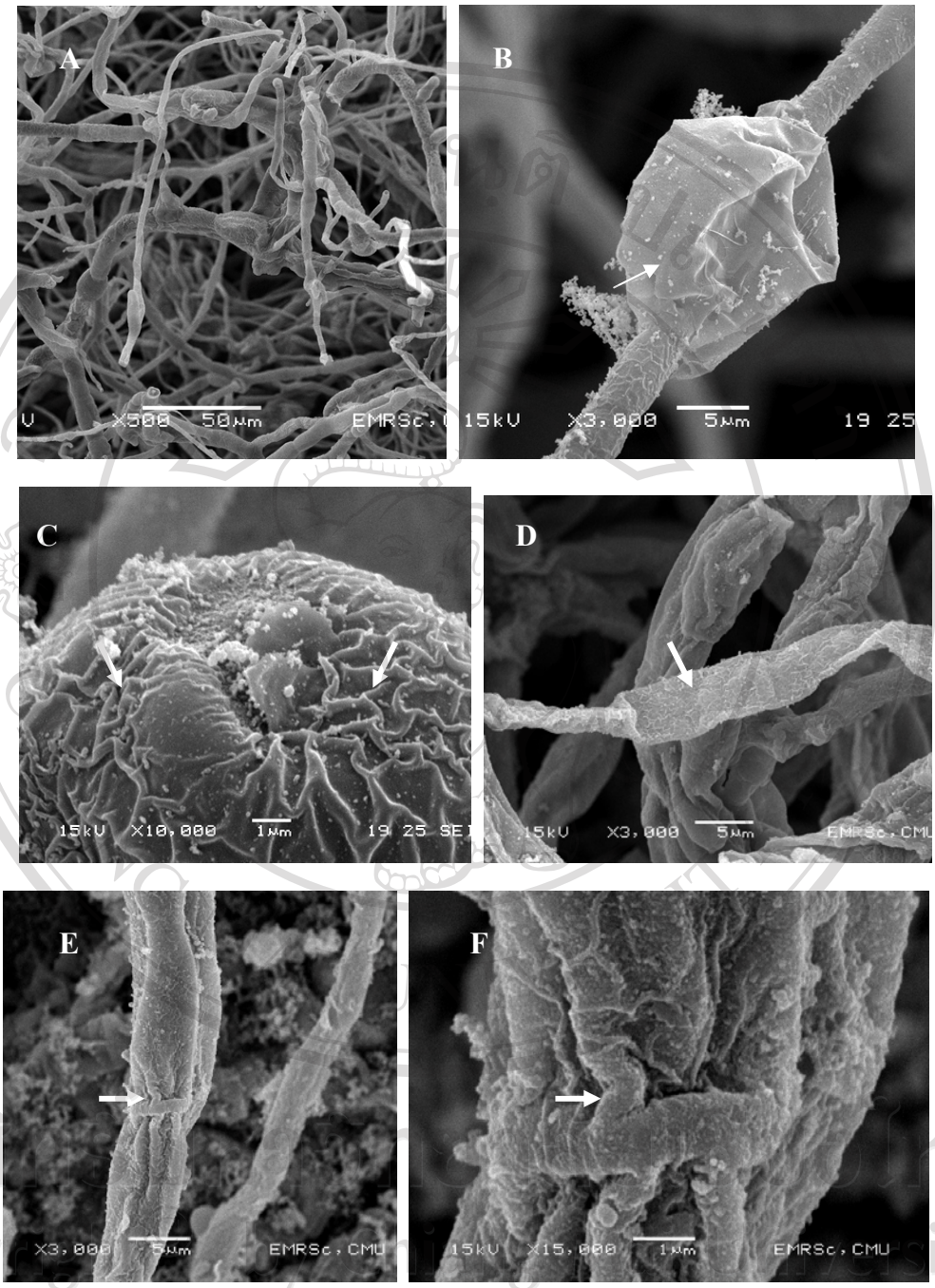


Figure 5.6 Scanning electron micrographs of *L. theobromae* mycelium in response to chitosan component. (A) - (F) 0.6% Cts, showing highly swollen hypha, evidence of an extracellular layer surrounding the hyphal cell and surfaced corrugation (arrow).

Citric acid mycelium

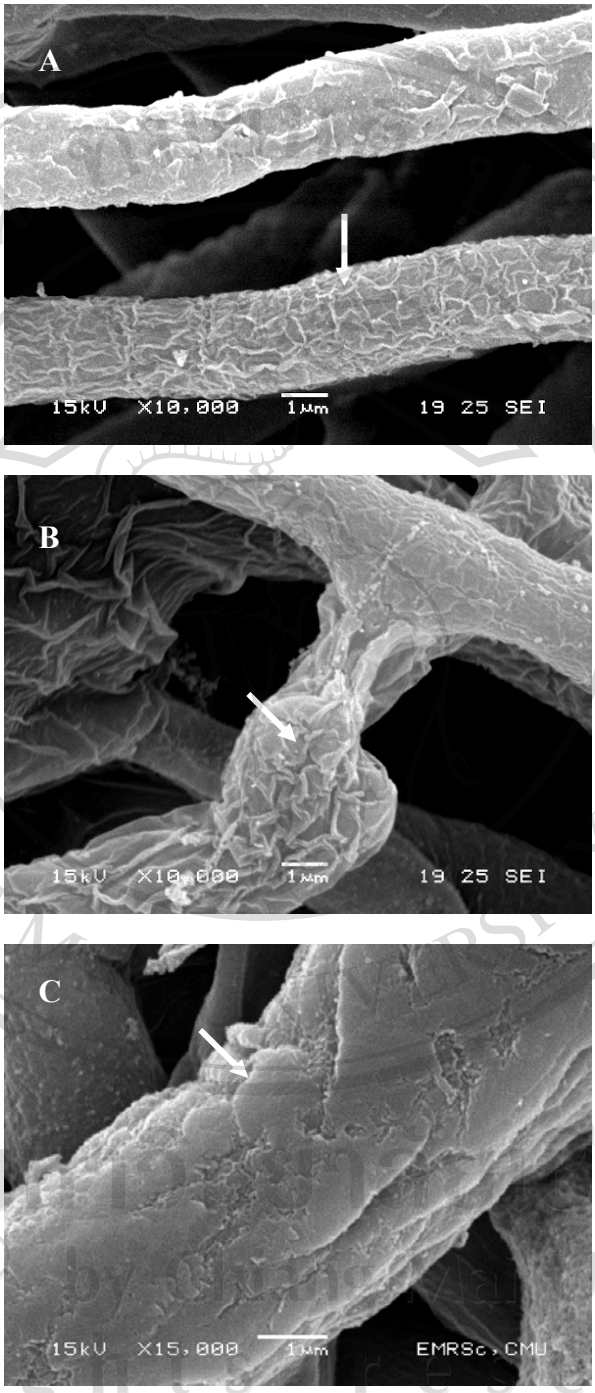


Figure 5.7 Microscopic structural changes in *L. theobromae* mycelium in response to citric acid component. (A-C) 1.0%CA, some hyphal surfaces are rough and swollen with sign of collapse (arrows).

Potassium sorbate mycelium

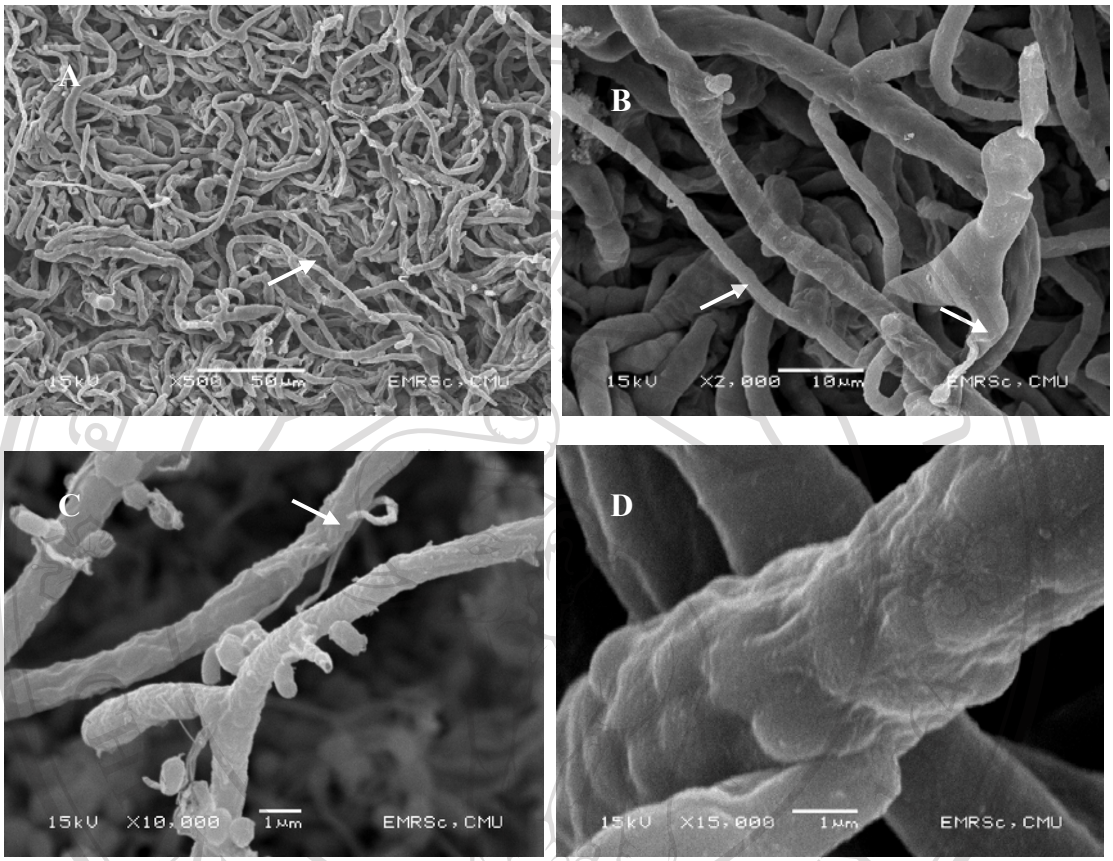


Figure 5.8 Microscopic structural changes in *L. theobromae* mycelium in response to potassium sorbate components. (A-D) 0.10%PS, some hyphal showing abnormal, swollen and small branches (less diameter hypha) (arrows).

5.4.2 Efficacy of integrated methods for controlling fruit rots development in *vivo* study

5.4.2.1 Disease development, SEM of infection site and sorbic acid in pericarp

In an *in vivo* trial, control fruit showed the highest disease severity score during storage (Figure 5.9b). The rotting areas (necrosis lesion) spread rapidly from stem-end down to the inner side of pericarp skin. Signs of dark brown area on both sides of pericarp were observed at hours 24. At h 48 dark brown areas on pericarp

increased and the aril was damaged. The aril became yellowish and off-flavor (Figure 5.10F; 5.11F). Suwanakood (2007) found that *L. theobromae* caused dark brown patches on the peel within 6 hours. The infected aril became rotten and the whole fruit was rotten within 48 hours in accordance to this study. The results showed that SEM a lot of mycelia are migrating from mycelia disk to inoculation site on stem-end fruit, mycelia are infecting to natural opening in stem-end (Figure 5.12a). This process hypothesized that this fungus could penetrate in stem end wounding (Pathak and Srivastava, 1967) or near stem end, lenticel on surface fruits (Holos and Divinagracia, 1969) and fruit lesion which spore generates germ tube. The fungus penetrate pass plant cell in intercellular and intracellular due to producing protease, pectinase and cellulose in a large content which digested cell wall (Nagaraja *et al*., 1971). In addition, it could produce pectinmethylesterase (PME) and polygalacturonase (PG) during infection. Wardlaw (1935) found that banana in either immature or mature showing the fungus penetrated in middle lamellae and produced pectinase around terminal hypha.

Application of coating component alone, such as 1.2% chitosan+ 3.0% CA as well as 0.30% PS showed high efficacy in controlling disease development within 48 hours when compared with 3.0% CA but did not differ significantly with the other treatments after hour 72 (Figure 5.9b). It has also been reported that chitosan coatings are not always more effective than synthetic fungicides, as demonstrated on litchi where chitosan delayed the infection process during 33 days storage, but was not as effective as TBZ in controlling rots (Zhang and Quantick, 1997). A similar lesser fungicidal effect of chitosan was reported on peaches, artificially inoculated with *M. fructicola* when compared to the fungicide prochloraz (Li and Yu, 2000).

One potential way to reduce this variability and improve efficacy is to combine compatible antifungal treatments that have different modes of action. Mixing CA in PS delayed disease development when compared with each compound alone (Figure 5.9b) and the results agreed with the *in vitro* laboratory experiment (Table 5.1, 5.2). Combination treatment using 0.3% PS + 1.2% chitosan + 3.0% CA showed lower disease development when compared with 0.3% PS + 3.0% CA. This suggested that growth of *L. theobromae* LP20 inoculated on the wounded stem-end were delayed in chitosan coating treatment containing PS in acidic condition. The

combination of PS and acid coating may provide greater, more reliable and broader spectrum disease control than that obtained using individual substances. These results were in accordance with those of Baldwin *et al*. (1996) who reported that Nature Seal (an edible coating, pH-2.5) containing PS significantly reduced mould count. Al Zaemey *et al*. (1993) also reported that addition of CA or PS in coating materials delayed lesion expansion of *Collectotrichum musae*. In addition, the highest sorbic acid content in pericarp using HPLC instrument of this treatment was detected compared to those treated without chitosan coating (Figure 5.9a). The degradation of sorbic acid content in pericarp exhibited negative exponential characteristic. Cts along with CA + PS could help to delay the rate of sorbic acid degradation in the pericarp (0.025 mg/kg/day) lower than that of CA + PS (0.040 mg/kg/day) and PS (0.041 mg/kg/day) (Figure 5.9a).

0.3% PS+1.2% Cts+3%CA	$y = 110.57e^{-0.0255x}$	$R^2 = 0.99$
0.3% PS+3%CA	$y = 70.565e^{-0.0401x}$	$R^2 = 0.98$
0.3% PS	$y = 71.141e^{-0.0414x}$	$R^2 = 0.99$

where y = sorbic acid in pericarp (mg/kg)

x = time (days)

Thus, edible coating films and coatings acts as surface retention agents, particularly when additives are included in the formulation and limit preservative diffusion in the food core (Cuppett, 1994; Vodjani and Torres, 1989). Chittenden *et al*. (2004) reported that dipping inoculated wood ship with fungal diseases in chitosan 1.5% mixed with GRAS compounds 0.5% to 1.5% (sodium benzoate, potassium sorbate and ascorbic acid) enhanced the performance of chitosan to delay disease attack. This combination was therefore also improving safety by inhibition or delay of the growth of microorganisms, due to their natural biocide activity or to the incorporation of antimicrobial compounds, allowing edible coatings to be widely used.

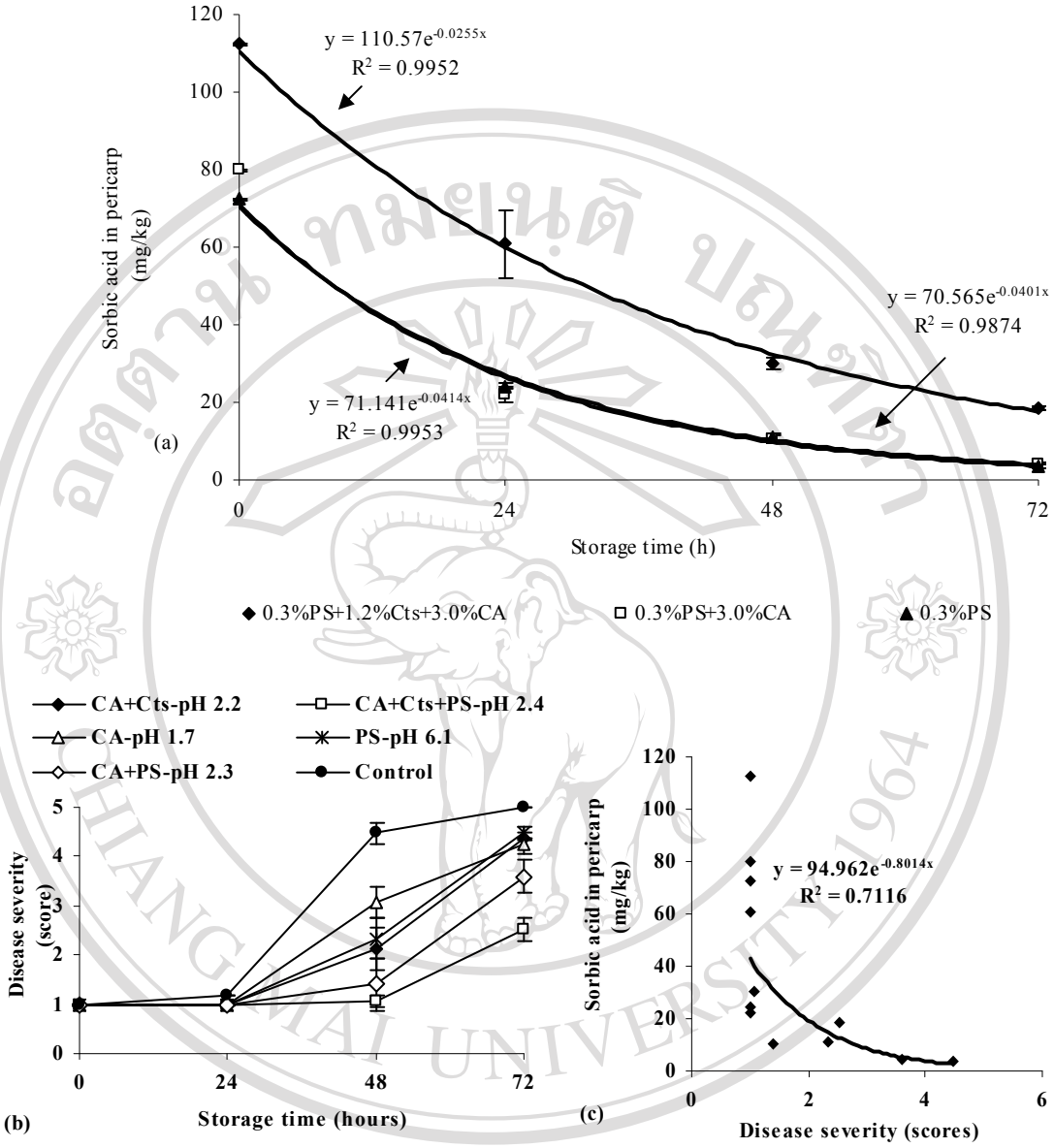


Figure 5.9 Effect of different combinations of coating components on sorbic acid degradations in pericarp (a), disease severity (b) and relation sorbic acid with disease severity (c) after inoculated with mycelial disk of *L. theobromae* on the wounds of stem end fruit 6 h prior to treatment and after storage at 25°C.

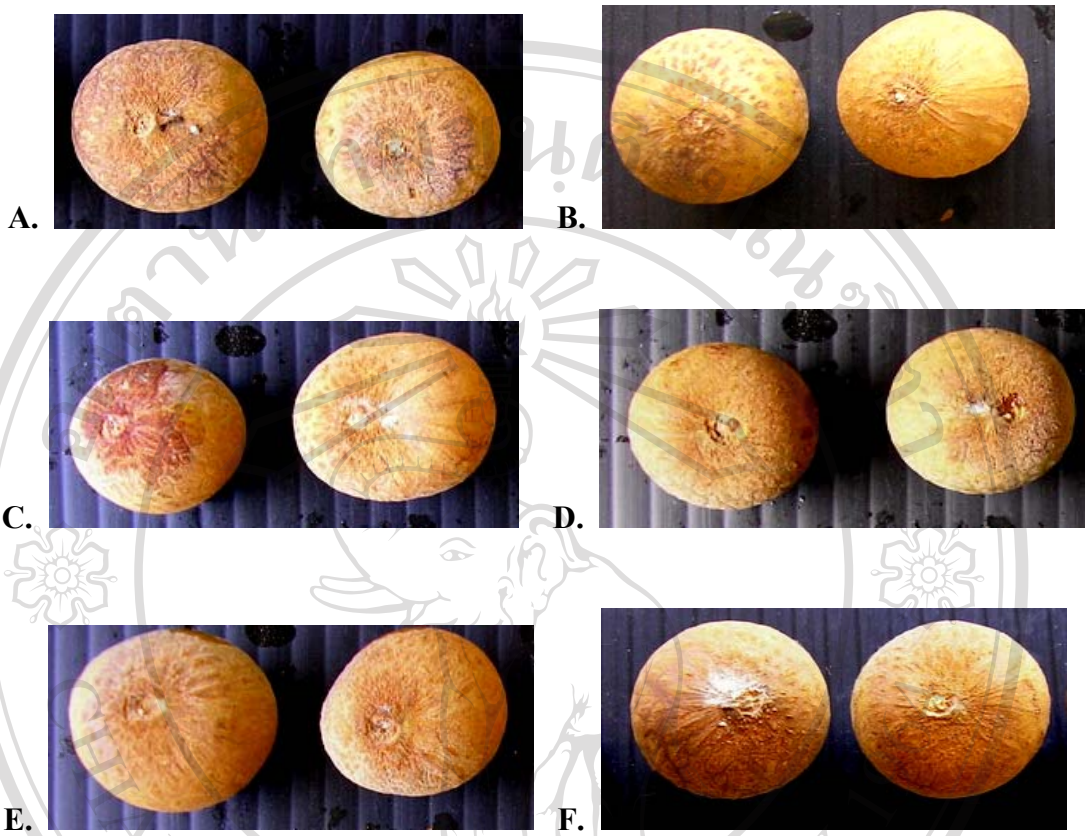


Figure 5.10 Effect of coating components and combined treatments against disease severity after inoculated longan with *L. theobromae* at hour 72. Necrotic lesion around the stem-end was observed.

A 1.2%chitosan+3.0%citric acid

B 1.2%chitosan+3.0%CA+0.3%potassium sorbate.

C 3.0%citric acid.

D 0.30%potassium sorbate

E 3.0%citric acid+0.3%potassium sorbate.

F Control

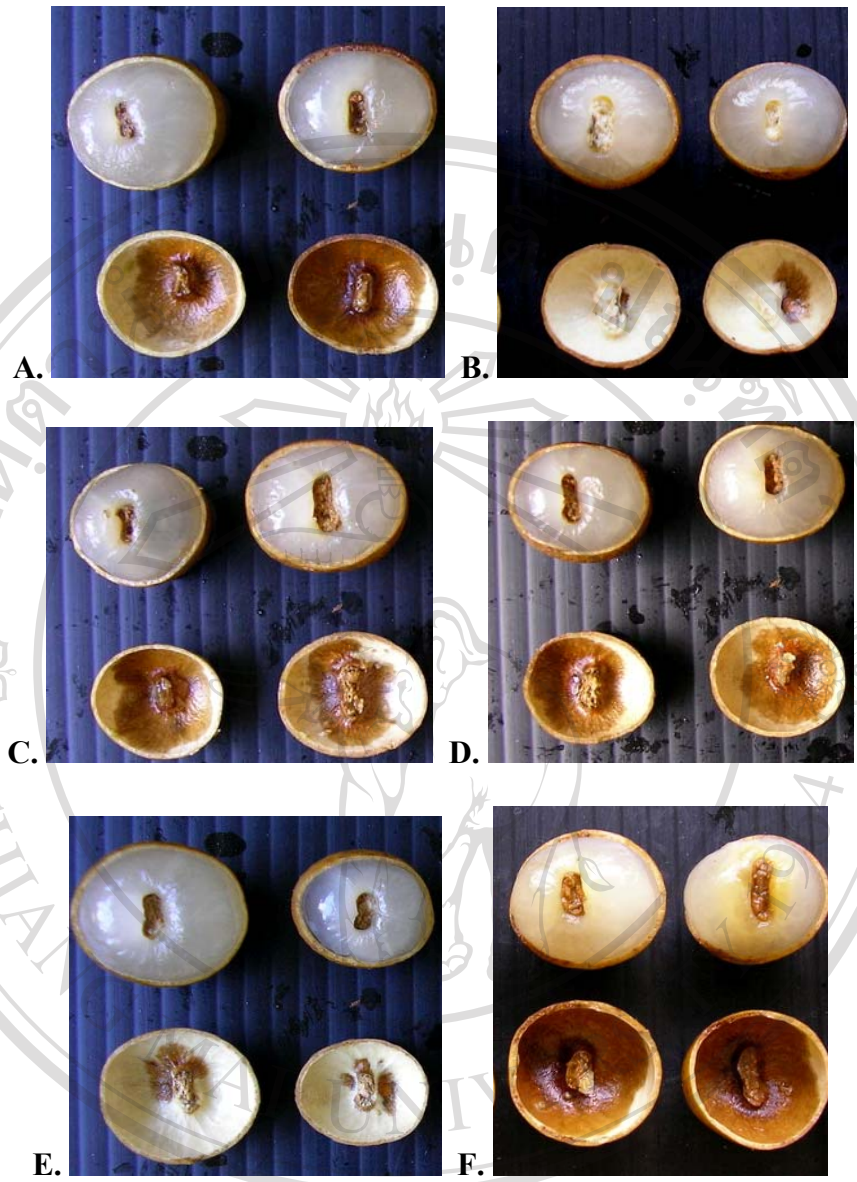


Figure 5.11 Effect of coating components and combined treatments against disease severity after inoculated longan with *L. theobromae* at hour 72. Necrotic lesion around the stem-end under pericarp was observed.

A 1.2%chitosan+3.0%citric acid

B 1.2%chitosan+3.0%CA+0.3%potassium sorbate.

C 3.0%citric acid.

D 0.30%potassium sorbate

E 3.0%citric acid+0.3%potassium sorbate.

F Control

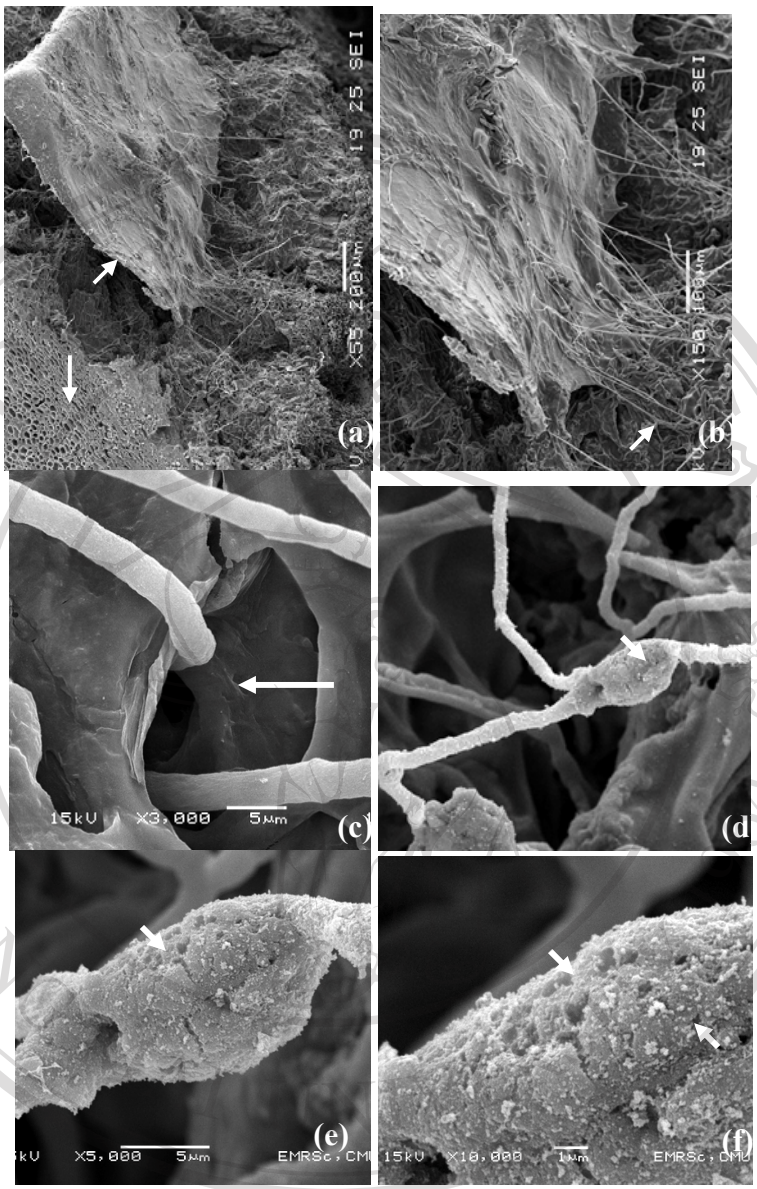


Figure 5.12 SEM micrographs showing the effect of chitosan coating incorporated with citric acid and potassium sorbate against mycelium morphological changes after inoculated longan with *L. theobromae* 24 hours. (A), (B) control fruits showing a lot of mycelia are migrating from mycelia disk to inoculation site on stem-end fruit; (C) mycelia are infecting to natural opening in stem-end; (D), (E) 1.2% chitosan+3.0% citric acid+0.3% potassium sorbate, some hyphal surfaces are swollen with some sign of collapse (F) many holes and dusts were observed on hyphal surface.

5.4.2.2 Mode of reactions of coating component on disease severity and PR protein as a defense mechanism

Control of decay development of longan inoculated before immersion for 5 min in 1.2% Cts+3% CA+0.3% PS at the pH 2.77 and 3.40 and 3% CA+0.3% PS-pH 2.4 separately, was improved significantly by the addition of antifungal substances in comparison with untreated fruit (Fig. 5.13a). Immersion in these combinations also reduced the number of decayed berries percentage to 0, 0 and 6.67%, respectively, while untreated fruit was the number to 100% during storage at 5 days (Fig. 5.13b). However, disease incidence percentage did not differ significantly among non-inoculated fruit treated with Cts + CA + PS (pH-3.3), Cts + CA + PS (pH-2.8) and CA + PS during 10 days as compared with control fruit (Figure 5.13a). The combination treatment using 0.3% PS + 1.2% Cts + 3.0% CA (pH 2.8) showed the lowest disease development when compared with Cts+CA+PS pH 3.3 and CA+PS pH 2.3 respectively. The control fruit showed the highest disease development during storage at 5 days (Figure 5.13a) and the results agreed with the last experiment (Figure 5.9b). The results revealed that increasing pH coating to 3.3 did not reduce the efficacy in controlling fruit decay. This finding agrees with the work of Sofos and Busta (1981), who found the best activity, was due to the undissociated form of the acid when pH was less than the pKa of 4.75.

Interactions between two factors on accumulation of the enzyme activity during storage were discovered (Table 5.3). The results indicated that treatments with or without inculcated fruit was related together with enzymatic accumulations. The increase of these enzymatic activities: chitinase and ß-1, 3-glucanase was predominantly related to wounding and pathogenic infection. The inoculated fruits prior treatments showed higher activities than non-inoculated fruits after 2 days (Table 5.3). PRs were induced after wounding and pathogenic infection and accumulation of specific enzymes such as chitinase and ß-1, 3-glucanase to protect infection of pathogens (Agrios *et al*., 1997). While non-inoculated fruits prior to treatments showed lower these enzymes activity, this suggested that chitosan along with PS + CA might induce only temporal profile activity and then degradation. They showed some variations during storage and therefore, they were not sufficient to protect disease development (Figure 5.13b, c; Table 5.3). From figure 5.13b, chitosan

along with PS + CA at two pH level after inoculated fruits or non-inoculated fruit temporally induced the highest chitinase activity as compared with control fruit during 3 days and then, all treatments were degradable and not significant. From figure 5.13c, chitosan along with PS + CA at pH 3.3 after with or without inoculated fruits could temporally stimulate the highest ß-1, 3-glucanase activity at 3.0 fold as compared with control fruit during 3 days and then, all treatments were degradable and not significant.

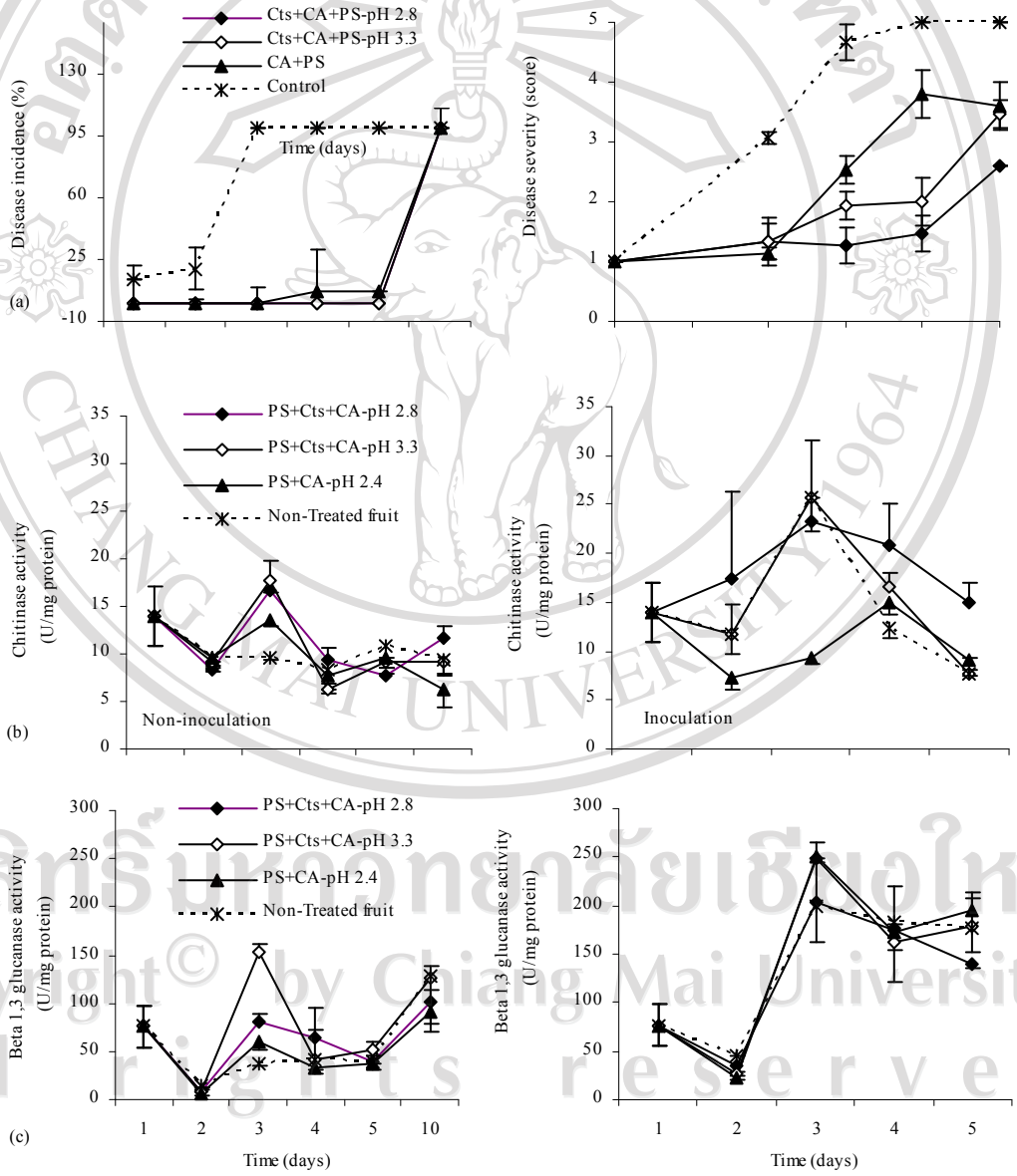


Figure 5.13 Chitinase activity (a) and β-1,3-glucanase activity (b) as a defense mechanism in longan pericarp after various treatments with or without *L. theobromae* LP20 inoculation.

The results indicated that chitosan coating in this experiment was not insufficient to induce PR-protein to control disease development. The result was accorded with the other reports (Jitareerat *et al*. 2008) and Kwanmongkhon (2008). El Ghouth *et al*. (1992) found that mechanisms of chitosan coating in reducing the decay of strawberries was related to its fungistatic property rather than its ability to induce defense enzymes such as chitinase, chitosanase and β-1,3-glucanase.

Many reports of chitinase and ß-1, 3-glucanase played a major role to directly attack or degrade chitin and ß-1, 3-glucan in cell walls of pathogens. Many produces; strawberry; mango; grape; and tomato (Bautista-Baños *et al*., 2006) was reported. A variation of enzyme activity might depend on variety of produce, stage of produce, technique in preparation, test of sample and temperature of extracting process.

Table 5.3 Interaction between inoculated fruit or without and chitosan coating or without on chitinase and β-1,3-glucanse activity during 5 days.

(1)	**Chitinase activity**			
	2	**3**	**4**	**5**
A = *L. theodiplodia* : Non-inoculated	9.24 b	14.9 b	7.85 b	9.27
Inoculated fruit	12.03 a	17.93 a	16.17 a	9.85
B = Treats: Cts+PS+CA pH 2.77	12.89	20.0 a	15.1 a	11.33 a
Cts+PS+CA pH 3.4	10.5	21.71 a	11.38 b	8.41 b
PS+CA pH 2.3	8.44	11.4 b	11.31 b	9.31 b
Untreated fruit	10.71	18.6 a	10.26 b	9.2 b
A	*	*	*	NS
B	NS	*	*	*
A x B	NS	*	*	*
C.V. (%)	26.2	16.9	12.8	15.84

Table 5.3 (Continue)

(2)	β-1,3-Glucanse activity			
	2	**3**	**4**	**5**
A = *L. theodiplodia* : Non-inoculated	9.83 b	82.03 b	44.97 b	42.15 b
Inoculated fruit	32.99 a	153.8 a	173.35 a	172.39 a
B = Treats: Cts+PS+CA pH 2.77	21.8 b	141.4 c	118.94	89.47 b
Cts+PS+CA pH 3.4	17.06 b	200.17 a	101.84	115.43 a
PS+CA pH 2.3	17.08 b	155.33 b	103.45	116.35 a
Untreated fruit	29.71 a	118.29 d	112.43	107.83 a
A	*	*	*	*
B	*	*	NS	*
A x B	NS	*	NS	*
C.V. (%)	18.9	7.07	22.26	11.93

1Same letters in the same column are not significantly different at 0.05.

2Cts = chitosan, CA = citric acid, PS = potassium sorbate

5.5 Conclusions

All coating components (chitosan, CA and PS) of coating material illustrated high efficacy in controlling *L. theobromae* as their concentrations increased but the effect was fungistatic. Abnormalities of hypha subjected to each of the components were found under microscopic observation. Mixing acidulant CA in all PS concentrations (0.1-0.3%) with or without chitosan could successfully improve the efficacy of fungicidal effects. 0.3% PS + 1.2% chitosan + 3.0% CA was the best treatment to control disease development *in vivo*. Concerning sorbic acid as active substance from PS component, Cts along with PS+CA maintained the highest amount of sorbic acid content in pericarp, known to be of great importance in longan resistance to this fungus. In contrast, decrease of disease severity and disease incidence did not related to the activity of chitinase and ß-1, 3-glucanase, but the increase of these enzymatic activities related to wounding and pathogenic infection. Chitosan could be used to a carrier of PS with CA stabilizer to protect longan fruit against postharvest disease.