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Effect of water-soluble chitosan on fungal anthracnose of *Colletotrichum musae*D1 *in vivo* and physico-chemical changes in banana fruits

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ABSTRACT: This study was carried out to evaluate the effect of various concentrations of water-soluble chitosan (WSC) on the growth of *Colletotrichum musae* D1, isolated from banana naturally infected by typical anthracnose, and the physico-chemical changes in banana fruits. In *in vivo* experiments, banana fruits inoculated artificially with 5µl *C. musae* D1 at concentration of 10^6 spores/ml were treated with WSC (0.5; 1.0; 1.5 and 2.0%). Experimental results proved that *C. musae* D1 is extremely sensitive to WSC andinhibitory effects increase with growing concentrations of WSC. The banana samples under WSC treatment showed a lower rate of infection and disease progression compared to the control samples (treated with distilled water). The concentration of WSC inhibiting the growth of anthracnose's lesion diameter on banana by 50% (IC_{50}) was 0.82%. Experiments in biochemistry indicated that the activities of the main defense-related enzymes in the peel, consisting of chitinase and β-1,3-glucanase and total phenolic content, were enhanced by 1.0% WSC treatment. In addition, WSC coating in banana fruits with a concentration of 0.5 - 2% at 25^{o}C retarded physico-chemical changes (weight loss, firmness, total titratable acidity, soluble solid content, respiration rate and ethylene production), accompanied by marked positive variations in sensory quality.

KEY WORDS: anthracnose, water-soluble chitosan, *Colletotrichum musae*, antifungal.

I. INTRODUCTION

Banana (*Musa cavendish*)is a popular fruit tree with high nutritional value grown in many tropical countries. However, banana is often susceptible to a range of diseases that result in major postharvest losses during transportation and storage. Caused by *Colletotrichum musae,* anthracnose is one of the common postharvest diseases in banana and the main cause for the short shelf life of banana. This pathogen invades the skin of immature fruits and remains in a "quiescent infection" state until the fruit ripens. Synthetic fungicides such as benlate (Benomyl), Imazalil and thiabendazole (TBZ) are the most commonly used method for controlling postharvest fungal diseases in banana. However, the overuse of these fungicides has resulted in toxic chemical residues on the fruit surface, the increasing chemical resistance of pathogens and serious problems for the environment[6].

Chitosan is a unique polycationic polymer of β-1,4, linked to D-glucosamine, chemically derived from crustaceans. This natural compound which is biodegradable and non-toxic has been recognized to be notably effective in maintaining the quality of harvested fruits and vegetables owing to its dual capabilities. It has an inhibitory effect on mycelial development and can spore germination of the fungal pathogens, change the morphology of the microorganism, and stimulate host defense responses [5], [12], [15], [16]. Chitinase and β-1,3-glucanase are important classes of pathogenesis-related enzymes and are elicited by pathogenic infection and in response to chitosan. Moreover, thanks to its ability to create semi-permeable membranes on fruits, chitosan can also limit postharvest changes and prolong the shelf life of fruits by reducing respiration intensity and water evaporation [5], [6], [12], [16].However, chitosan is difficult to use in practice due to its insolubility in neutral aqueous solution and their high viscosity in acid solution [27].

Recently, there have been considerable interests in converting chitosan to a water-soluble form [9]. Water-soluble chitosan (WSC) is more efficient in controlling pathogen development and stimulating numerous plant defense systems [9], [18]. We have previously reported the effectiveness of WSC against anthracnose caused by *C. musae*D1 in postharvest banana fruits *in vitro*[21]. To expand the potential application of WSC as an antifungal agent, this study was conducted to estimate the effects of WSC on the development of *C. musae*D1 in postharvest banana fruits *in vivo*, by evaluating the antifungal activity,defense mechanisms and physico-chemical changes.

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II. MATERIALS AND METHODS

A. Materials

The banana fruits (*Musa cavendish*AAA) were collected from a local farm in the Phu Vang District, Thua Thien Hue Province, Vietnam. Fruits were sorted by size, uniformity and without physical injury or disease infection. The fruit samples were washed with tap water, decontaminated by 70% ethanol and then cleaned with sterile water [24]. *C. musae* D1was isolated and identified from those infected banana fruits showing typical symptoms of anthracnose [21]. Water-soluble chitosan was prepared from chitosan powder (food grade with the degree of deacetylation (DD) 90 ± 5%, ash content ≤ 1%, As ≤ 1 mg/Kg, Pb ≤ 2 mg/Kg, Hg ≤ 0.5 mg/Kg) purchased from Hung Tien Co. Ltd, Vietnam, according to the method of Long et al. (2015) [20].

B. Methods

a. Antifungal assays of WSC against Anthracnose in vivo

The banana fruits were surface cleaned with 70% ethanol for 2 minutes and quickly flushed twice with purified water. Banana fruits were punctured by using a dissecting needle with holes of 1 mm in diameter and 2 mm in depth, and then 5 µl of conidial suspension of *C. musae* D1at 10^6spore/ml was pipetted into each lesion. After being air-dried for 1 hour, the selected fruits were dipped in WSC solutions of 0.5, 1, 1.5, and 2% for 1 minute, respectively. The fruit samples treated with sterile water were used as control. After drying in the air for 2 hours, the treated fruits were put in plastic boxes, moistened with sterile water and stored at 25^{o}C [24]. The lesion diameter was examined by a digital caliper every day for six days with three replicates for each treatment (5 fruits per replicate).

b. *Extraction and assay of Chitinase and β-1,3- Glucanase*

Banana fruits were collected and divided into 3 groups for evaluating defense-related enzyme activities with three replicates for each treatment (5 fruits per each replicate). The first group of fruit samples was inoculated with *C. musae* D1 and dipped into a 1% WSC solution. The second group was inoculated with fungus only, and the third group was uninoculated and untreated by WSC serving as a control group. All of the fruit samples were stored at 25^{o}C. Flesh portions encircling the lesions of fruit sample was taken every two days within 8 days.

The extraction of enzymes and the determination of β-1,3-glucanase activity were carried out following the method of Long et al. (2014) [19]. The activity of chitinase was analyzed based on the dinitrosalicylic acid method [8].

The content of total soluble protein was examined similar to the approach of Bradford [7].

c. Analysis of total phenolic content

Using the same fruit samples used for enzyme analysis, total phenolic content was determined using the Folin-Cicalteau method [13]. Total phenolic content was expressed as gallic acid equivalent (GAE per 100 g fresh weight).

d. Evaluation of physico-chemical changes in WSC-treated banana fruits

Banana fruits were dipped in distilled water (control) and WSC at a final concentration of 0.5, 1.0, 1.5, and 2.0% for 1 minute. Treated fruits were stored at 25^{o}C and samples were taken for evaluating levels of respiration and ethylene production, weight loss, firmness, total titratable acidity and content of soluble solids after 10 days of storage.

Each fruit was sealed in a 1500 ml plastic container for 3 h at 25^{o}C before headspace gas was withdrawn with a gas-tight syringe. Carbon dioxide and ethylene concentrations were determined by dual gas analyser (250/ICA) and ethylene analyser (ICA56) for CO_2 and C_2H_4, respectively.

The weight loss of banana fruit samples was measured by considering the differences between initial weight and final weight of current banana fruits divided by their initial weight (precision 10^{-4}g). The firmness ofthe fruit was recorded (N) with a penetrometer (Shimpo) using a 4 mm diameter tip. Soluble solids content of fruit was determinedby using a refractometer (Atago). A homogenous sample was prepared by blending the banana flesh in a blender. The sample was thoroughly mixed and a few drops were on the prism of a refractometer and a direct reading was taken by reading the scale in the meter as described in AOAC (1990) [2]. Total titratable acidity (expressed as citric acid %) of the fruit samples was identified by titrating 5 ml juice with 0.1 N sodium hydroxide, using phenolphthalein as an indicator[2].

The color of fruit peels according to the Cielab system was identified by a handy spectrophotometer (Nippon Densoku, NF 333). Changes in color intensity of fruit peels after 9 days of storage were assessed by the differences in parameters of L*, a*, b*, H^o and ΔE* ($\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$, $\Delta L^* = L_{treated}-L_{control}$; $\Delta a^* = a^*_{treated} - a^*_{control}$; $\Delta b^* = b^*_{treated} - b^*_{control}$).

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Statistical Analysis

The experiments were carried out by employing a completely randomized design. An analysis of variance was determined using the SAS version 9.1.3 (SAS Institute, USA) and differences between means were ascertained according to Duncan's Multiple Range Tests at $p< 0.05$.

III. RESULTS AND DISCUSSIONS

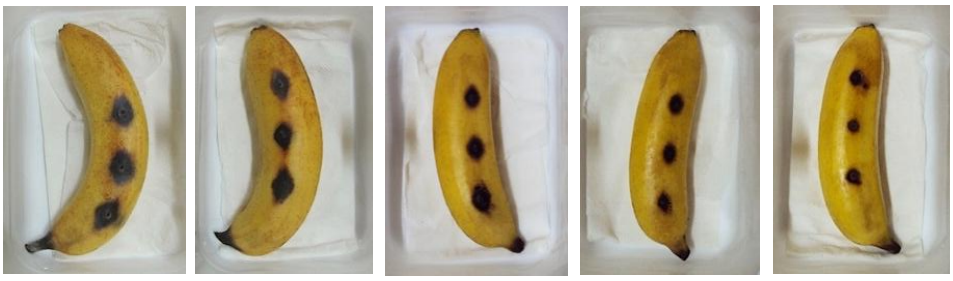
A. *In vivo* antifungal effect of WSC on anthracnose development

WSC inhibited anthracnose development based on the lesion diameter of banana inoculated with *C. musae* D1 (Table 1 and Figure 1). The earliest symptom of acthranose disease appeared white spot surrounded by rotten tissue after 1 day of inoculation at 25°C. This was observed after 2 days with 0.5 - 1% WSC; after 3 days with 1.5% WSC; and after 4 days with 2.0% WSC treatment. After 6 days, lesion diameters of WSC-treated banana fruits were much smaller than that of the control and were about 1.03 cm, 0.57 cm, 0.49 cm and 0.32 cm at 0.5%, 1%, 1.5% and 2% WSC, respectively, while that the control, 1.52 cm. The concentration of WSC inhibiting 50% (IC_{50}) of the growth of anthracnose's lesion diameter causedby *C. musae* D1on banana was 0.82%. Apart from inhibiting anthracnose development, WSC seemed as fruit darkening, respiration, weight loss and softening were retarded.

Table 1. The effect of WSC at various concentrations on lesion diameter of anthracnose disease on banana fruits

WSC (%)	Lesion diameter(cm)					PIRG (%)
	2 days	3 days	4 days	5 days	6 days	6 days
0 (Control)	0.07^{aE}	0.45^{aD}	0.77^{aC}	1.12^{aB}	1.52^{aA}	0
0.5	0.05^{aE}	0.25^{bD}	0.64^{bC}	0.92^{bB}	1.03^{bA}	31.84
1.0	0.04^{abD}	0.13^{cC}	0.44^{cB}	0.54^{cA}	0.57^{cA}	62.32
1.5	0^{bE}	0.07^{cdD}	0.35^{dC}	0.43^{dB}	0.49^{dA}	67.91
2.0	0^{bC}	0^{dC}	0.16^{eB}	0.28^{eA}	0.32^{eA}	79.06

For each column (row) mean values not followed by the same lowercase (uppercase) letter are significantly different ($p < 0.05$).



Control **0.5%** **1%** **1.5%** **2%**

Figure 1. Effect of WSC on lesion diameter of anthracnose on banana fruits inoculated with *C. musae* D1 after 6 days at 25°C

The results demonstrated that WSC had a clear inhibitory effect on the development of anthracnose disease caused by*C. musae* D1 in case they penetrate banana fruit in *in vivo* condition.WSC treatment was able to delay the onset of disease symptoms, to diminish the lesion diameter and to retard the development of anthracnose disease. However, the inhibitory effect of WSC on anthracnose development on banana fruits inoculated with *C. musae* D1 was less than that of in *in vitro* condition [21]. This result is consistent with that of previous studies on *C. capsici* [5],*C.gloeosporioides* [4], [16], [26].

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Although the accurate antifungal mechanisms of chitosan and its derivatives *in vivo* are still unknown, the fungal inhibition activity of WSC can be explained by some modes of action. Firstly, there is the presence of large amounts of amino groups in the structure of WSC, which can interact with the negatively charged radicals on the surface of fungal cell wall. This reciprocal action may cause the change in permeability properties of fungal cell membrane and bring in the leak of intracellular electrolytes or additionally restrict the penetration of materials which lastly leads to the death of the fungus [3], [15]. Another proposed mechanism for the fungal inhibition activity of WSC may be due to the contact of diffused hydrolysis substances with DNA of fungi when WSC permeates through the fungal cell. This effect results in the prevention of mRNA and protein synthesis [17]. Lastly, the withdrawal of essential metal ions, trace elements of important nutrients by forming a chelate with WSC is also believed to be an explanation of inhibiting the conidial germinationand the growth of fungi [3], [17].

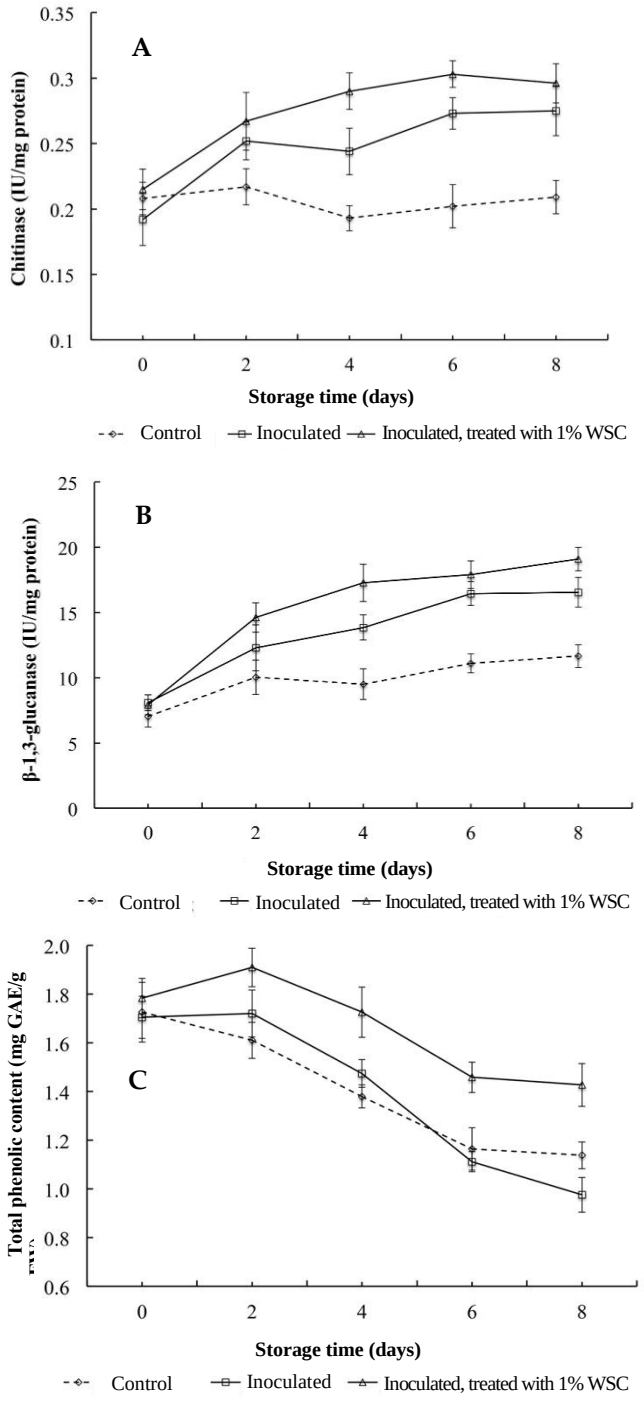

Figure 2. Effects of WSC on activity of chitinase (A), β-1,3-glucanase (B) and total phenolic content (C)of banana fruits. Vertical bars represents ±SE of means for three replicates.

B. Effects of WSC on defence-related enzymes and total phenolic content in banana fruits

The inhibitory effect of WSC on anthracnose was further characterized in terms of the activity of key defense-enzymes in the flesh around the inoculated area of the fruit. Chitinase activity increased in response to pathogen inoculation (Figure 2.A). WSC further increased chitinase activity. This was evident after 2-8 days of storage. On the eighth and last day of storage, uninoculated and untreated fruit had a chitinase activity of 0.209 IU/mg protein while the inoculated fruit without and with WSC had 0.275 IU/mg protein and 0.292 IU/mg, respectively. A similar trend was obtained for β-1,3-glucanase activity (Figure 2.B). Inoculated WSC-treated fruits had the highest activity, followed by the inoculated, untreated fruit, while the control (without inoculation and WSC) had the lowest.WSC also increased the total phenolic content relative to that of untreated fruit with and without pathogen inoculation (Figure 2.C). The highest phenolics content was obtained on the second day of storage and was about 1.910 mg GAE/g FW for inoculated, WSC-treated fruit. With the advancing storage period, phenolics content quickly decreased. On the last day of storage (8 days), the total phenolic content in uninoculated and untreated fruit was about 1.138 mg GAE/g FW while the inoculated fruit without and with WSC was about 0.975 mg GAE/g FW and 1.427 mg GAE/g FW, respectively.

The protection of fruits against infection by fungal pathogens is largely due to a structural defense system and the activation of a highly-coordinated enzymes that help to prevent the spread of pathogens. In company with peroxidase, polyphenol chitinase are regarded as core enzymes which directly combat pathogenic fungus in plant disease-resistance systems [15]. The amount of these antifungal hydrolases plays a major role in the defense function against pathogens by corrupting the

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fungal cell wall since β-l,3-glucan and chitin are the main structural constituents of the cell wall of most pathogenic fungus [11].Besides, cuticular penetration of *Colletotrichum* spores into the surface of fruit is associated with the induction of production of extracellular cutinase capable of hydrolyzing the cuticle layer of the epidermic cells. During the infection process, the fungus also secretes pectolytic enzymes, which are capable of decomposing the non-soluble pectic compounds and causing tissue disintegration and cell maceration [6]. In addition, phenolic compounds which are secondarily metabolized, are significant, not only as preformed antifungal agents, which represent the first agent of defense against pathogenic fungi along with the cuticle at the surface of fruits, but also as phytoalexins formed in plant tissues in response to the infectious process. The development of the pathogens can be diminished or reduced only when phenolic compounds at the cite of the pathogen invasion are accumulated at a high level [23]. The *in vivo* result of our study demonstrated the antifungal effectiveness of WSC in controlling anthracnose disease. WSC treatment was able to delay the onset of disease symptoms, to diminish the lesion diameter and to retard the development of anthracnose disease. Furthermore, WSC stimulated defense-related enzymes and elicited the raise in the total phenolic content of banana fruits.

C. Effects of WSC on physico-chemical changes in WSC-treated banana fruit

Although WSC treatment had a little influence on the weight loss after 3-4 days of storage, there was a significant difference between the fruits coated with WSC and uncoated fruit samples (control) after 6 days of storage (Figure 3.A). The effect of WSC treatment on the firmness was somewhat different from that of the weight loss. Firmness of fruits after 9, 10 and 12 days of storage significantly decreased compared with the control fruits(Figure 3.B).

Win et al. (2007) reported that chitosan coating minimized the weight loss of stored banana and its combination with heat treatment showed the lowest respiration [28]. Jitareerat et al. (2007) also showed that chitosan formed a coating film on the outside surface of the mango that effectively retarded the loss of water [16]. The weight loss in banana during storage is due to water movement from fruit pulp to peel, release of volatiles containing ethylene, and carbon dioxide by respiration, and evaporation of water from the peel during ripening [25].

The ripening of banana fruits is characterized by loss of firmness due to cell wall digestion by pectinesterase, polygalacturonase and other enzymes [6]. Higher fruit firmness in WSC coating fruits could be attributed to the permeability property of the coating and its effects on the fruits, and this coating provided a better way to reduce the water evaporation. Overall better retention of firmness in chitosan coating fruits as compared to untreated can be explained by retarded degradation of insoluble proto-pectins to the more soluble pectic acid and pectin [23].

The content of soluble solids increased rapidly in the control fruits and was significantly different from the WSC treated fruits after 6 days of storage. After 10 days of storage, while the content of soluble solids in the control fruits increased to 24.01%,the content of soluble solids in the fruits treated with 0.5% and 2% WSC reached 20.31% and 13.62%, respectively(Figure 4.A). Thetitratable acidity tended to increase, reaching the highest value at 4 days of storage (in non-treated fruit and 0.5% WSC treated fruit) and 6 days of storage (1%, 1,5% and 2% WSC treated fruits) then decreased gradually. WSC treatment slowed down the reduction of titratable acidity compared to the control samples (Figure 4.B).

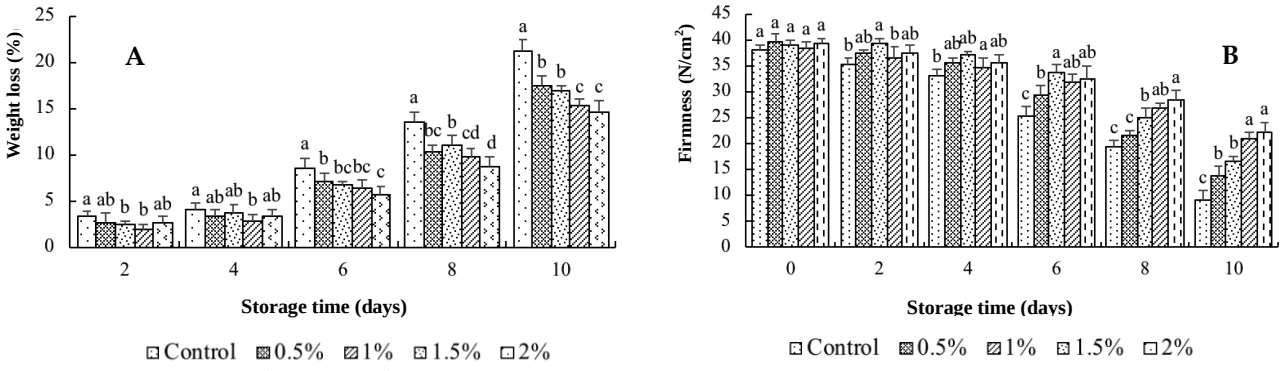

Figure 3. Effects of WSC on the weight loss (A) and firmness (B) of banana fruits. Vertical bars represents ± SE of means for three replicates.

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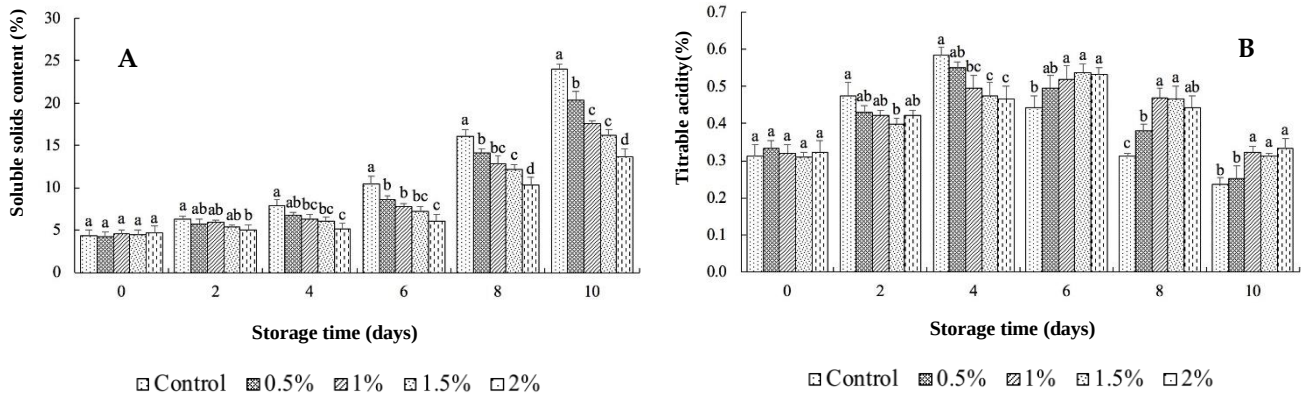

Figure 4. Effects of WSC on the content of soluble solids (A) and titrable acidity(B) of banana fruits. Vertical bars represents ±SE of means for three replicates.

The higher levels of total soluble solids in the fruits coated with chitosan may be due to protective O_2barrier reduction of oxygen supply on the fruit surface which inhibited respiration [1]. Du et al. (1997) reported that application of chitosan coating decreased total soluble solids of fruits caused by a decline in the amount of carbohydrates and pectins, partial hydrolysis of protein and decomposition of glucosides into sub-units during respiration [10].Win et al. (2007) also showed that chitosan coating significantly delayed ripeningin terms of firmness and soluble solids of stored banana.While the decrease of titrable acidity during storage time demonstrated fruit senescence [28],the change in acidity might be due to the effect of chitosan treatment on the biochemical condition of fruit and a slower rate of respiration and metabolic activity [2]. This was probably because the semi-permeable chitosan film formed on the surface of the fruit might have modified the internal atmosphere, thus retarding ripening [28].

The respiration intensity and ethylene production are considered important indicators of determining the shelf life of banana fruit. WSC treated fruits with a concentration of 0.5% and 1% reached the respiration peak after 6 days of storage, while it took 8 days of storage for 1.5% and 2% WSC treated fruits to reach the peak(Figure 5.A). The effect of WSC treatment on the ethylene production was slightly different from that of the respiration rate.WSC treated fruits with a concentration of 2% had the highest value of ethylene production after 8 days of storage (Figure 5.B).

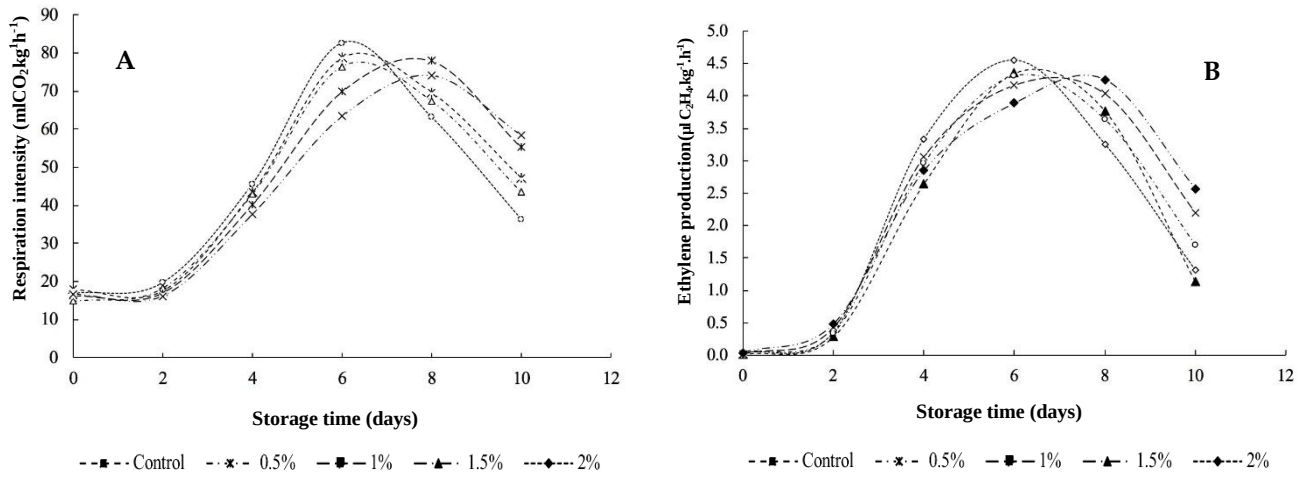

Figure 5. Effects of WSC on respiration rate (A) and ethylene production (B) of banana fruits.

The variations in respiration could be due to water loss percentage in the banana fruit, which caused a reduction of preclimacteric period, stimulation in ethylene production, respiration at preclimacteric stage, and change in quality [25]. Jitareerat et al. (2007) showed that chitosan coating significantlyreduced respiration rate and ethylene production

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accompanied by the decline in the ripening process [16]. Mapbool et al. (2011) also reported that chitosan coating provided an excellent semipermeable barrier around the banana fruit, which modified the internal atmosphere and suppressed ethylene evolution, thus reducing respiration. Moreover, the reducing sugar content and the total soluble solids of coating fruit were lower than with the untreated fruit, which suggests that the coated fruit synthesized reducing sugars at a slower rate, through the slowed metabolism [22]. In our study, at a higher treatment concentration of WSC, the respiration rate and ethylene production were lower and, thus, reduced the ripening of the fruit.

WSC treatment had also slowed down the ripening of banana fruits by altering the color intensity of the peels. The values of L and a* on banana fruits slightly changed but the values of b* and H varied considerably, corresponding to the color change of fruit peels after 10 days of storage. The effect of WSC treatment on fruit color change through the difference of ΔE*was only observed in 1%, 1.5% and 2% WSC treated fruits (Figure 6).

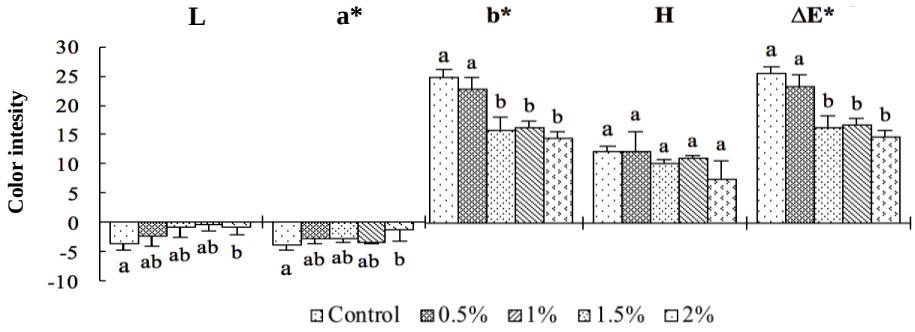

Figure 6. Effects of WSC on the color intensity of banana peels after 9 days of storage. Bars with different letters are significantly different at $p < 0.05$.

Color changes could be attributed to the breakdown of chlorophyll in the banana peel throughout the storage duration [25]. Some research results have reported changes to the color of the fruit peel after chitosan application, which were confirmed by visual appearance. Papaya fruit treated with chitosan underwent a light change in peel color, as indicated by the slower increase in lightness and chroma values, as compared to uncoated fruit. The delay of color development for the papaya fruit treated with 1%, 1.5% and 2% chitosan might be attributable to the slow rate of respiration and reduced ethylene production, which leads to delayed fruit ripening and senescence [5]. During storage, chitosan coating also delayed color changes in banana [22], [28], mango [1] and tomato [14].

IV. CONCLUSION

From the present studies, we conclude that applying a WSC coating can effectively control anthracnose in banana fruit *in vivo* with concentration-dependent antifungal effects and can prolong the quality attributes of banana fruit. Inhibition effect of anthracnose disease caused by *C. musae* D1 appears to be the result of multiple actions of WSC. Moreover, our study indicates that WSC retarded physico-chemical changes and enhanced positive variations in sensory quality of banana fruit. These findings suggest that WSC may be recognized as a feasibly effective alternative to artificial fungicides for postharvest disease control and safe preservatives for extending the shelf life of banana fruits. However, more studies are necessary to confirm the mechanism of WSC action against postharvest anthracnose of banana caused by *C. musae*and other *Colletotrichum* species.

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