

The role of hot water treatment and chitosan coating in controlling a latent infection of *Colletotrichum musae* on banana var. Mas kirana

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Received:

April 09, 2018

Accepted:

October 31, 2018

Published:

December 31, 2018

Abstract

Anthraco disease due to a latent infection of *Colletotrichum musae* has seriously impacted on bananas decay. This case caused a serious number of economic losses facing in both local and international trades. To overcome this problem, hot water treatment and also chitosan coating have been developed in minimizing this fungal infection during postharvest period. In this research, a number of green mature local premium banana called bananas var. Mas kirana were inserted into hot water at 44, 46 and 48°C subsequently for 5, 10, 15 and 20 minutes. Another treatment also carried on through a wide range of chitosan solutions from 2.5, 5 and 10 g/L soaked for 2 and 4 minutes. As a single treatment, hot water soaking at 48°C for 20 minutes gave the best effect on handling the fruit maturity until 18 days of storage. This level treatment could also suppress the lesion only 2.4 cm and also minimized the severe fruit-damaging level until 22 days of observation. Whereas, chitosan coating through dipping in 5 g/L chitosan solution for 2 minutes gave the best result on controlling latent fungal infection in-vitro. However, this treatment only minimized fruit-damage in medium level until 16 days of storage. Furthermore, the combination treatment of hot water treatment at 48°C for 20 minutes followed by chitosan coating at 5 g/L through dipping in 2 minutes gave the most proper result in terms of handling of fruit ripening until 23 days of storage. Another result proved this combination treatment significantly eliminated the length of lesion up to 0.16 cm and suppressed the fruit-damage in mild level. However, there were no significant differences among the yellow mature of untreated and combination-treated bananas observed from tests results of color characteristics, pulp strength and total dissolved solid content.

Keywords: Hot water soaking, Chitosan, *C. musae*, Banana var. Mas kirana

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Introduction

As one of the banana (*Musa* spp.) exporting countries, Indonesia has cultivated banana var. Mas kirana as a premium commodity. This commodity has a prosperous side to trade in the international market due to its yellowish brightly skin, sweet taste and pleasant flavor. However, a massive infection of *Colletotrichum musae* as a pathogenic mold can cause

banana decaying which impacts a significant economic loss.

This mold is the main vector of anthracnose disease in banana fruits. Infection occurs when germinated fungal spores transform to appressoria. Then, those appressoria attach on the surface of young banana in the orchard. The symptoms are detected at ripening stage, particularly when appressoria convert into infected hyphae which trigger to a rapidly quiescent



anthracnose (Mirshekari et al., 2012). In several cases, there are found this disease has severely deteriorated fruit during transporting from fruit storage facilities to ripening room prior to being displayed for purchasing (Chillet et al., 2007).

Hot water treatment (HWT) is an alternative solution to solve the previous problem. This treatment has become more popular due to the raising consumer's awareness of negative impacts of fungicide residues. A number of previous studies have proven that the treatment can prevent the spreading of post-harvest diseases caused microorganism contamination on several horticultural products (Afek et al., 1999; Fallik, 2004). Water has acted as the heat transfer medium for shorter periods at higher temperatures (Jacobi et al., 1996; Aborisade and Ojo, 2002). In another study, Acedo Jr. et al. (2001) reported anthracnose disease infecting banana var. Latundan and Saba were successfully impeded through dipping in hot water medium at 47-52 °C for 10-20 minutes. HWT has also related to induction of cellular tissues resistance in Cavendish banana var. Gros Michel with the result that the fruit has not been blackening when it has been stored at 4 °C for 4 days (Promyou et al., 2008).

A different option has been offered through the application of chitosan coating. As a natural substance, chitosan is encouraged as the excellent coating material which has controlled post-harvest diseases on several tropical horticultural commodities (El-Ghaouth et al., 1992). This substance has been reported to inhibit tomato gray and blue mold rot in papaya (Eryani et al., 2009) and also to control anthracnose in mango (Jitareerat et al., 2007). Others studies mentioned a significant lowering level of infection and severity caused by *Rhizopus stolonifer* subsequently on papaya, peach and tomato coated by chitosan (Han et al., 2004; Wang et al., 2007; Meng et al., 2010). It has prolonged the shelf of life of numerous fresh fruit commodities, such as banana, grape, strawberry, and raspberry (Bautista-Banos et al., 2006; Meng et al., 2008). Therefore, it has been used as natural preservatives for the fresh and processed food (Wilson et al., 1994).

The study about the selected temperature and time exposure of HWT to control anthracnose disease in banana var. Mas Kirana and also its effect to the shelf of life of banana based on pathological quality are rarely published. This information is important to ensure the phytosanitary status of banana which is ready to be exported. In another side, the effect of

chitosan coating in suppressing the latent infection of *C. musae* is an alternative option to decrease the fruit-damaging level during the storage period. The combination treatment on the selected dosage of HWT and chitosan coating is critical to strengthen the fruit stability and prevent mold contamination without degrading the fruit quality. This study was carried out to investigate the scientific evidence obtained through a series of experiments using HWT and chitosan coating and the selected combination of HWT and chitosan coating in handling banana maturity and controlling anthracnose disease.

Material and Methods

Plant materials

Mature green bananas var. Mas Kirana mostly freed from lesion were harvested from Parakansalak orchards, the 8th of national plantation company, Sukabumi region-West Java Province, Indonesia. The weight of a bunch of bananas were around 1.5-2 kg and the selected fruits had similarities in color and maturity. Overall research stages were conducted from January to December 2017.

Identification of *C. musae*

Isolate of *C. musae* was obtained from the anthracnose infected fruits using a method explained by Anthony et al. (2004) and pure cultures were cultivated on PDA cultures. Moreover, morphological characters, such as: colony diameter, size and shape of conidia, were identified based on the previous methods described by Photita et al. (2005) and Jinyoung et al. (2002).

Preparation of inoculums

Nine days old PDA cultures of *C. musae* were used for inoculation. Then, the conidia suspension was achieved through a thinning dilution using sterilized distilled water until the colony density reached 10⁵ conidia/ml. This step was processed under the laminar flow cabinet (LA2-6AX, Esco Class II BSC, PA, USA) with the inflow and downflow velocities were subsequently 0.53 and 0.33 m/s. The final suspension was inserted into several tubes sizing two milliliters.

In-vitro viability of *C. musae*, heat treatment, and chitosan coating for fruit

For in-vitro assay, the tubes containing conidia suspension were soaked in stirred thermostatic bath (GD 100, Grant, Cambridge, UK) at 44, 46 and 48 °C, respectively for 5, 10, 15 and 20 minutes. Then, the



tubes were cooled by using distilled water at 21-23 °C for half of each of thermal exposures times. In a different way, 50 ml of conidia suspension were poured into beaker glass, then each of 2.5, 5 and 10 g/l chitosan solution were subsequently added to beaker glass. In overall, those treatments were done in triplicate. Both of the tubes and beaker glass were stored at room temperature for a night for incubation. Soon after incubation was completed, the slide of conidia spores of *C. musae* was prepared based on the method developed by Khan et al. (2001). There were three replicates of agar plates from each different kind of treatment. Observation was taken place under compound microscopes (Axio Scope, A1 Pol, Carl Zeiss Microscopy, NY, USA) from six different view sides at 40 times magnification. Germinated conidia spores was calculated as the relative ratio of the abnormal spores to the entire ones. In a different assay, 400 µl of chitosan solutions and 10 ml of liquid PDA were poured into several petri dishes. The work was continued by shaking the dishes until it was formed the homogeneous solution. Pure isolate of nine days *C. musae* was taken out by using a cork borer, then it was planted in the middle of a mixed and solidified medium. Initial diameter of colonies were 5 mm. Observation of developed colonies diameter was carried on daily until all of the colonies on the control fulfilled the dishes. Colonies diameter was measured in two directions based on a method described by Khan et al. (2001). Inhibition of fungal growth was empirically counted from an equation developed by Hendricks et al. (2017).

In another way, the green mature bananas were treated by heat treatment using the same temperatures and exposures times as taken place previously. Soon after the hydro-cooling process accomplished, the fruits were stored at 16-18°C and RH 60-65%. In different treatments, others bananas were soaked on the chitosan solutions at each concentrations 2.5, 5 and 10 g/ml for 2 and 4 minutes. Those two-type treatments were reworked three times. After that, the fruits were dried in room temperature for almost four hours, then the fruit were kept on the storage room. A series of daily observations had taken place to compare the development fruit maturity, the size of lesion and the fruit damaging level during storage. For the full yellow mature bananas (maturity scale = 6), the fruits then analyzed in three different characteristics such as: peel and pulp color, pulp hardness and total dissolved solids content.

The selected combination treatments

Combination treatment given to green maturity bananas was based on the previous result, which was strongly able to minimize the growth of *C. musae*. Those treatment were hot water soaking at 48°C for 20 minutes followed by hydro-cooling process at 22°C for 10 minutes and coating in 5 g/l chitosan solution through dipping for 2 minutes. All of experimental units were repeated three times. After the two main processes were completely done, both of treated and untreated bananas were stored at 16-18°C and RH 60-65%. Then, daily observations for almost a month were implemented to investigate the same parameters as had been listed for the previous fruits treatments.

The development of fruit maturity

All of bananas were observed to analyze the given treatment effect to fruits ripeness. A series of daily observation were conducted to recognize the symptoms of ripen fruits based on the standard of fruit color alteration developed by SH Pratt & Co, Luton-UK (Nannyonga et al., 2016). Those scales were explained as follows: 1 = all green; 2 = green with traces of yellow; 3 = more green than yellow; 4 = more yellow than green; 5 = yellow with traces of green; 6 = all yellow; 7 = all yellow with brown speckles. The observation was stopped when the fruits maturity had reached seven-scale as the initial fruits decay indicated by the appearance of brown spots on the surface of fruits peel.

Length of lesion on the surface of fruits peel

Appearance of lesion as the beginning indication of fruits decay was consistently observed when fruit maturity had reached seven-scale. The length of observed lesion was daily measured and stated in centimeters. The following accretion of lesion on each replicated samples were calculated as the ratio between the aggregate of lesion sizing on each of fruits fingers and the overall of the infected fingers caused by those lesion.

Fruits-damaging level

Fruit damage was assumed as the ratio of total infected fingers to the overall bananas fingers. The criteria was based on the method developed by Pramyou et al. (2008). The deterioration level during storage period was described using a scale from 1 to 5 where 1 = no fruit deterioration observed; 2 = mild deterioration (1-20% fruit affected); 3 = moderate deterioration (21-50% fruit affected); 4 = severe deterioration (51-80%



fruit affected); 5 = very severe deterioration (81-100% fruit affected).

Peel and pulp fruit color

Measuring of lightness and chromaticity was carried on by using a Konica Minolta Colorimeter (CR-13, Osaka-Japan). On-site internal calibration was done prior to analyze all of fruits samples. Both of the peel and pulp of six-scales ripen fruits were attached on the edge of glass-side detector, then the data series of L, a, and b were read from the instrument monitor.

Fruit hardness level

All of six-scale mature bananas were measured their pulp hardness by using the fruit hardness tester (KM 1/5- Fujiwara Scientific, Japan). The cone probe was used to measure the pulp strength. The probe was pressed until it penetrated through the pulp, then the value was read in units of kgf. Measurements were repeated in three times.

Content of total dissolved solid

The six-scales ripen bananas were blended. The fruits juices were dropped into the lens of refractometer (PAL-1, Atago, Tokyo-Japan). The value was read on the monitor and it was expressed in units of °Brix. Measurements were done as many as three replications.

Statistical analysis

Data from the previous experiment were statistically analyzed through analysis of variance (ANOVA). Calculation of means ratings were determined using Duncan's multiple range tests at $P \leq 0.05$. In the verification test, the effect of selected combinations treatments and control were statistically analyzed by using paired t-test at the same P-value. All experimental data were obtained from three times replication.

Results and Discussion

Microscopic features of *C. musae*

The purified colonies of *C. musae* were clearly seen orange on PDA. These conidia were mostly ellipsoid, hyaline, ranging from 11-17x4-6 μm (Fig.1. A-B). This fact had several similarities with the previous research reported by Abd-Elsalam et al. (2010) stating the colonies color changed from white to orange during the incubation period. It was also stated the similarity shape of conidia ranging from 12-17x4-8

μm (13.5 x 6.0 μm).

In the same perspective, Jinyoung et al. (2002) confirmed the alteration color of those colonies was also followed by appearance of several black, acervulus-like masses developed on the culture plates after 10 days-incubation at room temperature. This research also reported appressoria were formed in both round and irregular shape from germ tubes, ranging from 6-11x5-10 μm and the setae were negatively found on both the lesion and the cultures.

Effect of hot water soaking to conidia germination of *C. musae*

The treatment significantly suppressed in-vitro conidia germination. In general, the declining of germination rate had clearly been observed, when the levels of temperature and exposure time were drastically added. The treatment at 48°C for 20 minutes had given the most significant impact on decreasing of conidia germination, which was almost a half than the value obtained in the control (Fig. 2).

This finding was positively correlated with the previous research reported by Schirra et al. (2000) expressing that hot water treatment had a significant role on reducing the germination rate of several fruit decay-molds. Furthermore, this treatment had also greatly impacted on losing activity of conidia germination so that infection of several kind-pathogen mold were able be well-controlled.

Effect of thermal treatment in controlling the post-harvest disease caused by *C. musae*

Different with the previous result, all of the treatment conducted less than 20 minutes were not sufficiently impact on prolonging fruit ripening and therefore those fruits were more susceptible to latent infection of *C. musae* during storage period (Table 1). As the storage periods were getting longer, the lesion size appeared on the peel surface were much bigger. This finding was a main cause of the initial fruit-damage indicated by the spreading out of brown spots encircling the whole fruit.

Otherwise, all of the thermal treatment carried on at 20 minutes were properly extending fruit maturity. Comparing with the others, the treatment at 48°C for 20 minutes was able to give the best effect on controlling the peak maturity until 22 days. The same effect was also obviously seen on reducing the lesion size, then reducing the portion of infected fruit and minimalizing the fruit decay subsequently until 2.4 cm, 33.33% and 1 as the lowest rating of fruit



deterioration (Fig. 3. A-B). This result was a quite sophisticated, particularly for suppressing latent infection of *C. musae*.

The previous works mentioned that hot water treatment induced tissue resistance of fruit impacting on reduction of pathogen growth (Barkai-Golan, 1991; Lopez-Cabrera and Marrero-Dominguez, 1998; Follett and Sanxter, 2001; De Costa and Erabadupitiya, 2005; Mansour et al., 2006). Moreover, hot water treatment conducted above 40°C affected in reducing microbial growth on fruit peel compared than untreated fruit (Dissanayake et al., 2015). This study also confirmed that the shelf-life of the treated fruits were longer than that in the control. Furthermore, for the thermally treated fruits were able to maintain the structure integrity of pulp upon the microbial growth occurred later (Kamdee et al., 2009). Therefore, this treatment had a good potential to be applied on preventing anthracnose disease, particularly during postharvest period.

Effect of *in-vitro* chitosan on suppressing the growth of *C. musae*

All of chitosan solutions in a wide range concentrations from 2.5 to 10 g/l were significantly able to decline germination power. These solutions also critically contributed on lowering spores diameter size and suppressing relative growth of *C. musae*. In overall, those results were quite promising, particularly in relating chitosan as a potential bioactive agent for controlling pathogen mold (Table 2).

In the columns, means followed the same small letter are not statistically different by the Duncan test at 5% probability.

Based on the latest result, 5 g/l of chitosan solution had the highest ability on minimalizing germination rate and also the spore growth of *C. musae*. This solution seemed to be the most significant bioagent to eliminate the mold population until 80%. In addition, this trend also drastically impacted on the smallest size of spore diameter reaching 18 mm. Previous studies reported that chitosan solutions in a certain range of concentrations were able to inhibit microbial growth through the change of cell permeability due to interaction between positive charge from chitosan molecules and negative one from microbial cell membranes causing leakage of proteinaceous and other intercellular constituents (Leuba and Stossel, 1986; Papineau et al., 1991; Sudarshan et al., 1992; Jinasena et al., 2011)

Effect of chitosan coating on detaining fruit deterioration

Comparing with the untreated fruits, bananas treated chitosan coating in many different concentrations from 2, 5 to 10 g/l given by soaking for 4 minutes were less successful in controlling the peak-fruit ripening. Those treatments were also failed in suppressing early lesion. Therefore, the fruit-damage in medium level until 50% was early occurred (Table 3). A slight different from the latest result, fruits treated Chitosan coating in many different concentrations from 2,5 to 10 g/l through soaking in 2 minutes showed more resistant to a massive pathogen infection. After dipped in chitosan solution at 5 g/l, those fruits got lesion in the smallest size, but there was no significant different in controlling the severity of fruit decay at the end of storage. (Fig. 4. A-B).

In contrast with control, fruits treated chitosan coating in two different concentrations i.e. 2.5 and 5 g/l had a better internal resistance in facing a massive infection in a last couple days. Moreover, fruits treated chitosan coating on 5 g/l had given a better result in terms of the shortest length of lesion observed in the last storage periods. This result had a positive relation with the previous work reported by Xiangchun et al. (2012) described that chitosan as a natural antifungal agent was able to activate a number of different enzymes relating with the defense of fruit tissue so that pathogenic mold infection could be managed.

This work confirmed that combination treatment of HWT at 48°C for 20 minutes followed by chitosan coating at 5 g/L through soaking in 2 minutes seemed to be prosperous in handling *C. musae* infection in bananas. Therefore, the combination treatment were tried on confirmatory test.

Influences of combination treatment in handling *C. musae* infection on fruits

Compared with each of single treatments, the combination treatment gave the best result in terms of handling fungal infection on fruits, therefore the fruits were able to be handled in longer storage periods. Until 22 days, all of the treated fruits had not reached the peak maturity. This result impacted on improving tissue resistance of fruits, so that fruit decay and lesion on fruit peel could be totally eliminated (Fig. 5. A-B). The most significant improvement had been obtained particularly in preventing fungal infection due to the most of fruit given the combination treatment were not excessively ripe in almost three weeks. The lesion size appeared on the treated fruits scattered on minimum

rate. Therefore, the fruit damage was sharply decreased. Compared with control, the lesion on the combination-treated fruit began to appear after three weeks of storage with a minimum impact to fruit-decay. This result also proved that there was a synergistic relation between HWT and chitosan coating in terms of enhancing the fruit resistance against fungal infection, particularly *C. musae* until the last period of storage.

Influences of combination treatment to physical characteristics of fruit quality

There were no significant differences on color, the strength of pulp and also total dissolved solid observed on both the control and the combination-treated fruits. Based on analytical result, the lightness and intensity of chromatic both on peel and pulp were statistically almost the same (Table 4). A slight difference was found only on b-value, where the combination treatment significantly impacted on lowering the yellowness intensity.

In the same columns, means followed the same small

letter are not statistically different by the paired-t test at 5% probability.

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The same trends were also obtained from both strength of fruit flesh and total dissolved solid tests. There were no statistically differences observed between the two treatments (Table 5).

In a different perspective, Prasanna et al. (2007) previously mentioned the selected lower storage temperature also contributed on maintaining fruit texture stability in a longer period. Through this process, the rate of pectin hydrolysis was able to be minimized so that the flesh could not easily shrink. Meanwhile, a different study conducted by Sampio et al. (2007) described that a ripening process had the very close relation with the conversion of polysaccharide into several molecules of simpler sugar. The effect of this conversion was quite clear, particularly when those fruits achieved the top of ripening.

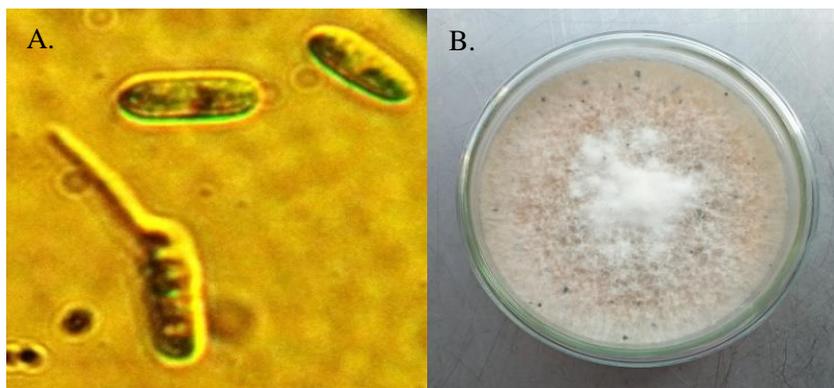


Fig. 1. Morphological characters of *Colletotrichum musae* were isolated from the anthracnose wound on commercial bananas var. Mas Kirana: A.) Germinated-oval conidia (green arrow) with appressoria (red arrow); B.) Colony of *C. musae* on PDA medium incubated for 24 hours at room temperature

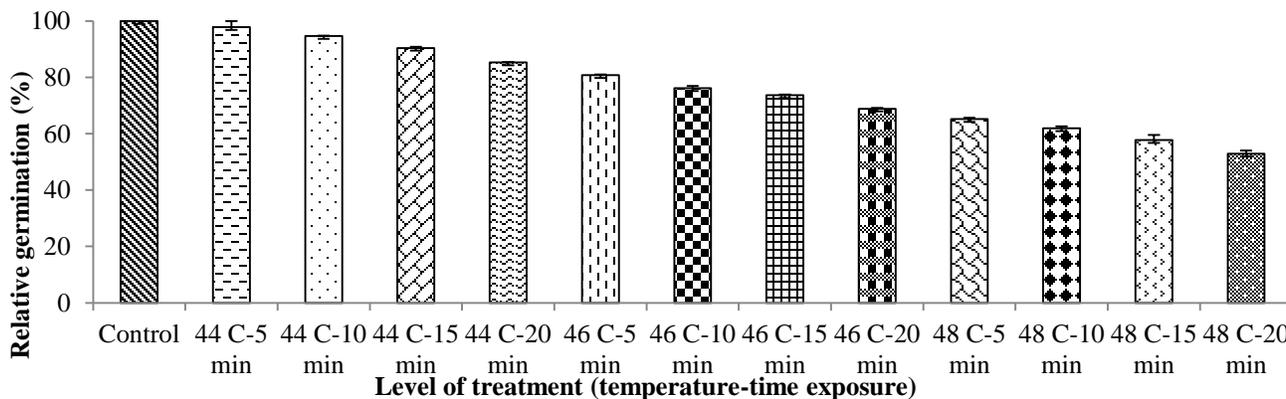


Fig. 2. Reduction of in-vitro conidia germination of *Colletotrichum musae* treated with hot water treatment observed after a night incubation at room temperature. The value was presented as mean ± standard error. The best level of treatment was shown by asteric sign indicated the least number of normal conidia growth counted on the prepared slide

Table 1. Critical periods for achieving fruit maturity (fm), initial lesion size (ils) and medium-fruit damage level (mfd) for bananas thermally treated at 44, 46 and 48°C subsequently for 5, 10 and 15 minutes mostly gave shorter handling period, particularly in suppressing fungal infection and fruit decay than the data obtained from control.

Thermal treatments	Critical periods (days)		
	Fm	ils	Mfd
Control	20	18	20
44°C-5 minutes	18	14	17
44°C-10 minutes	19	14	19
44°C-15 minutes	20	13	17
46°C-5 minutes	18	16	18
46°C-10 minutes	19	13	16
46°C-15 minutes	21	16	18
46°C-5 minutes	22	18	20
46°C-10 minutes	22	15	19
46°C-15 minutes	21	15	21
48°C-5 minutes	22	18	20
48°C-10 minutes	22	15	19
48°C-15 minutes	21	15	21

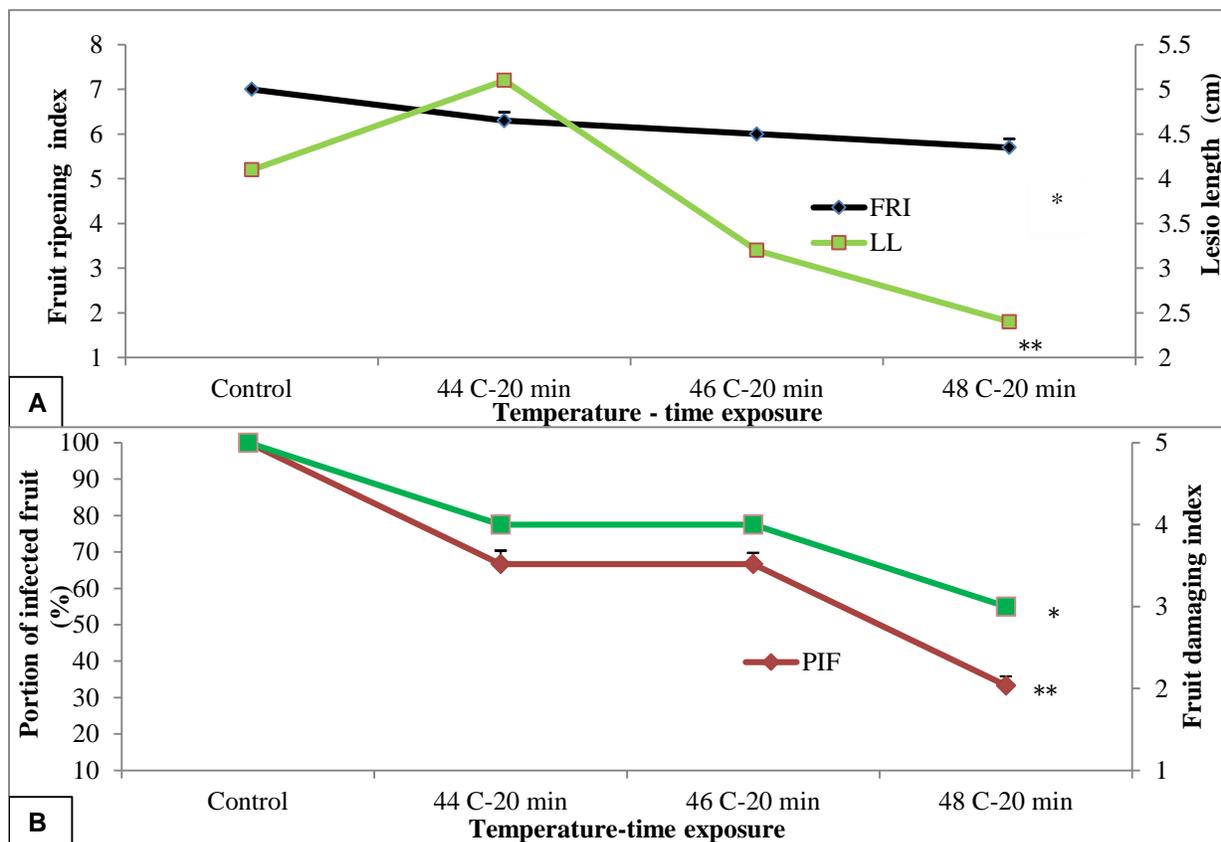


Fig. 3. Effects of raising temperature for 20 minutes exposure on handling fruit maturity and minimizing lesion spread on peel surface observed after 22 days storage. The observed parameters were stated as: A.) fruit ripening index (FRI) and lesion length (LL) and B.) portion of infected fruit (PIF) and fruit damaging index (FDI) . The data were presented as mean ± standard error. The best level of treatment was shown by the asteric and the double ones indicating respectively on the least influence subsequently on FRI, LL, PIF and FDI values.

Table 2. Eliminating of in-vitro spores growth parameters after treated using a wide range of chitosan solutions. The data were collected after a night incubation at a room temperature and presented as mean ± standard error. The best treatment was shown in the first sequence describing the least of those growth parameters.

Concentration (g/l)	Germination power (%)	Diameter spores (mm)	Relative growth (%)
5	47.83 ± 0.03 a	18 ± 0.58 a	20 ± 0.58 a
2.5	49.43 ± 0.34 a	21 ± 1.16 a	24 ± 1.16 b
10	58.14 ± 6.98 b	29 ± 1.73 b	33 ± 1.73 c
0 (control)	100 ± 0 c	90 ± 1.73 c	100 ± 0 d

Table 3. Contradictive effects to critical attributes on fruits treated a wide range of chitosan solutions soaked in four minutes. Those fruit damaging indicators were seemly observed earlier than those on the control.

Concentrations (g/l)	Critical periods (days)		
	Fruit ripe	Appearance of lesion	Medium fruit decay
0 (control)	20	18	20
2.5	19	15	19
5	18	15	17
10	16	15	13

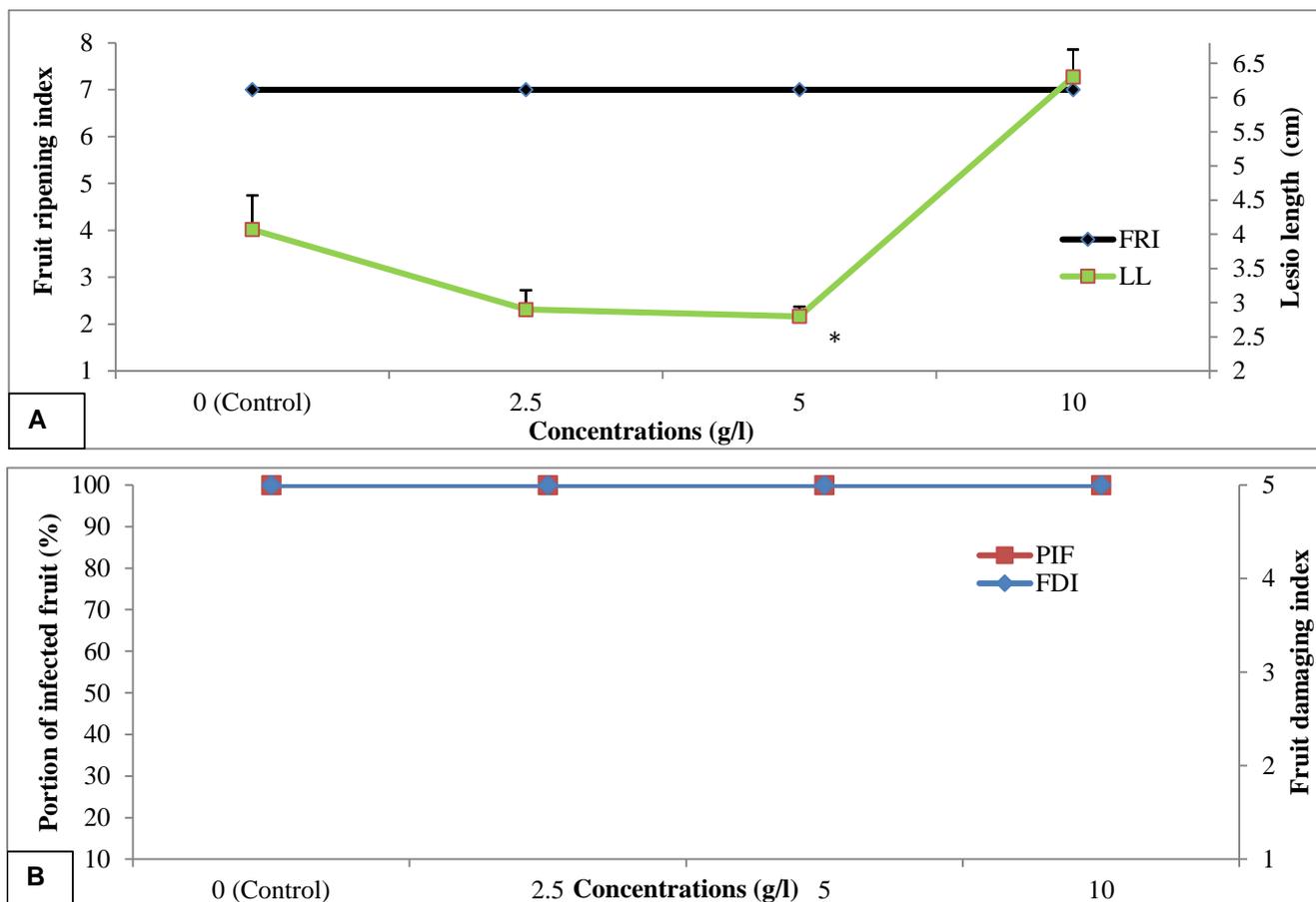


Fig. 4. Roles of limited chitosan concentrations on handling the fruit ripe and minimalizing the spreading of lesion size after 22 days of storage. The observed parameters were stated as: A.) the fruit ripening index (FRI) and lesion length (LL) and B.) portion of infected fruit (PIF) and fruit damaging index (FDI). The data were presented as mean ± standard error. The best level of treatment was shown by asteric sign indicating on the least influence LL values.

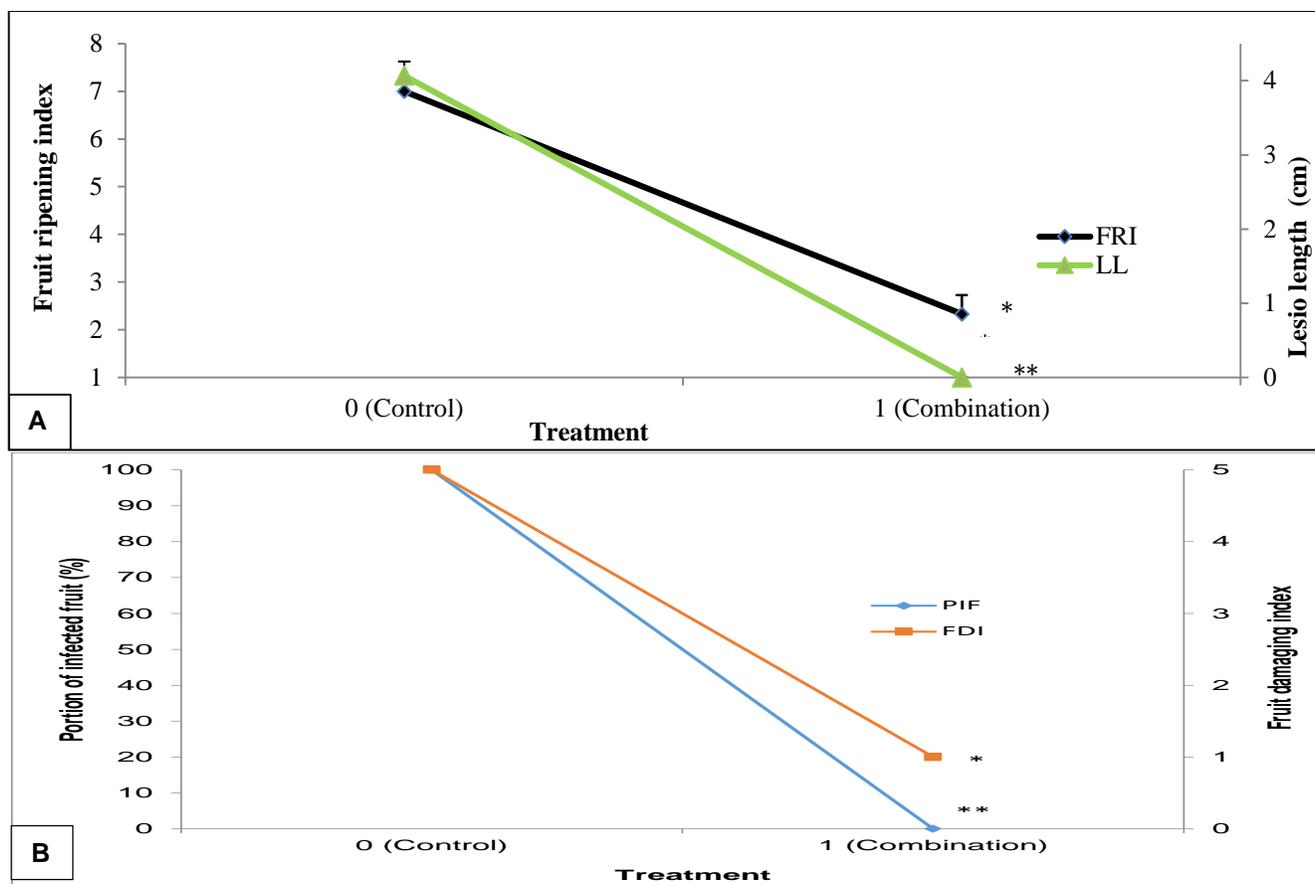


Fig. 5. Comparison effects between selected combination treatment and control on handling fruit ripe and eliminating lesion on peel fruit after 22 days of storage. Observed parameters were stated as: A.) fruit ripening index (FRI) and lesion length (LL) and B.) portion of infected fruit (PIF) and fruit damaging index (FDI). Data were presented as mean \pm standard error. The best level of treatment was shown by asteric and the double ones indicating respectively on the least influence respectively on FRI, LL, PIF and FDI values

Table 4. Color characteristics (L, a and b values) observed on ripe fruits.

Treatment	Peel			Pulp		
	L	a	b	L	a	B
Control	68.3 \pm 2.28 a	8.6 \pm 0.77 a	51 \pm 1.55 b	70.7 \pm 1.57 a	4.63 \pm 0.07 a	36.3 \pm 0.52 b
Combination	68.3 \pm 0.78 a	9.5 \pm 0.65 a	48.5 \pm 0.26 a	73.6 \pm 3.42 a	4.67 \pm 0.06 a	34.7 \pm 0.04 a

Table 5. Pulp strength and total dissolved solid observed on ripe fruits.

Treatment	fruit flesh strength (kgf)	total dissolved solid ($^{\circ}$ Brix)
Control	0.25 \pm 0.002 a	10.97 \pm 0.22 a
Combination	0.26 \pm 0.002 a	12.8 \pm 0.59 a

Conclusion

It is concluded that hot water treatment at 48°C for 20 minutes could be used to control a latent infection of *C. musae* on Mas Kirana banana instead of using

chitosan as a natural fungicide. The combination of hot water treatment at 48°C for 20 minutes followed with soaking in 5 g/l chitosan solutions for 2 minutes is recommended to alter agrochemical use in postharvest of banana. Further study should be taken place for



investigating the effects of both of each single and combination treatments in stimulating responses of several kinds of essential enzymes which are important in strengthening tissue resistance against fungal infection.

Acknowledgment

The authors would like to appreciate the financial support of Indonesia Agriculture Quarantine Agency, Ministry of Agriculture and the 8th National Plantation Company for its technical supports.

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