Effects of Hot Air Treatment on Softening of Pulp during Ripening in Banana Fruit

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Effects of Hot Air Treatment on Softening of Pulp during Ripening in Banana Fruit

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List of abbreviations

ACC: 1-Aminocyclopropane-1-carboxylic acid

ACO: ACC oxidase

ACS: ACC synthase

BSA: Bovine serum albumin

DNS: 3,5-Dinitrosalicylic acid

DW: Distilled water

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid

GC: Gas Chromatography

H°: Hue angle

HSPs: Heat shock proteins

PG: Polygalacturonase

PL: Pectate lyase

PME: Pectin methyl esterase

PMSF: Phenylmethane sulfonyl fluoride

PVP: Polyvinylpyrrolidone

SAM: S-adenosyl-L-methionine

SDA: Sodium deoxycholate acid

SDS: Sodium dodecyl sulfate

TBA: 2-Thiobarbituric acid

MET: Methionine

CHAPTER I

General Introduction

1.1 Banana's taxonomy

Bananas are classified into genus *Musa* of family Musaceae. The genus contains three sections (Paull and Duarte, 2011) in which all edible bananas belong to Eumusa section (Now Musa (Wong et al., 2002)). The genomes of two species in this section in which *Musa acuminata* and *Musa balbisiana*, are defined as A and B represents dessert type and wild type, respectively (Office of the gene technology regulator, 2008). The most commercial banana cultivars are triploids A-genome, AAA group and production of Cavendish subgroup covers 47% in the world market, whereas plantain and cooking bananas with AAB group and ABB, respectively share lower percentage (FAO, 2003).

1.2 Banana's market

Bananas are counted as one of the most valuable commodities around the world in 2013. The total production was 106 million tons from more than 50 countries, and the volume is continuously increasing over ten years (FAOSTAT, 2016). Asia is a region where banana production is the largest, as India, China and Philippines are the top 3 producers (Figure 1.2.1, 1.2.2). However, a global trade of banana exportation is mainly supplied by Ecuador where a quantity of banana exported in 2013 was the highest in the world at 5 million tons (Figure 1.2.3). European Union of 27 member states (EU 27 ex. Int.) is the largest importing area with the biggest shared of 29% or 3,894 million dollars in the global market (Figure

1.2.4). Regarding company, the Chiquita, Fresh Del Monte and Dole are predominant world bananas exporters whose market shares started to decrease from 2002, while a value of small suppliers' shares began to increase to more than 50% (FAO, 2003). In the Asian region, Philippines is the biggest banana exporter while Japan is the leading importer. According to figure 1.2.4, Japan imports accounted for 6 % of bananas.

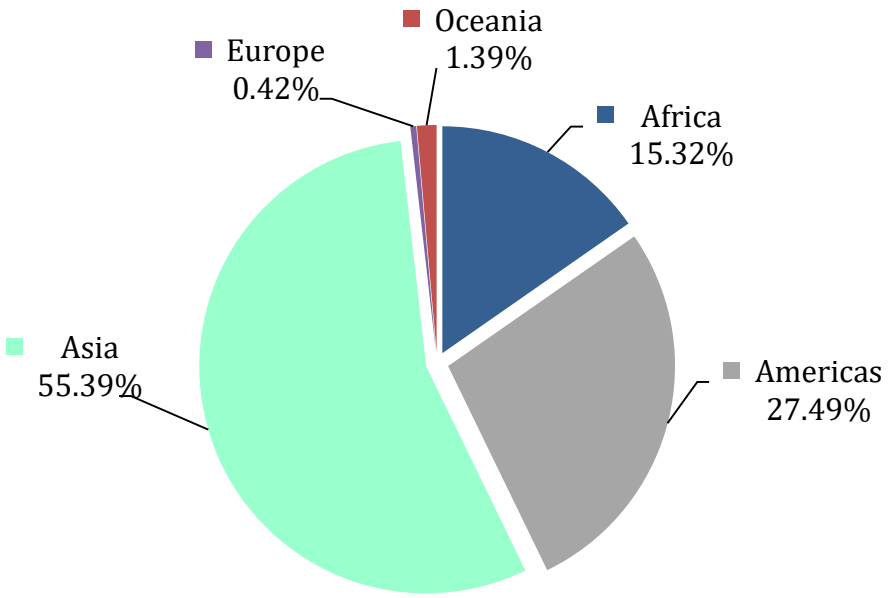


Figure 1.2.1 Banana production share by region year average in 2004 - 2014,

Source: FAO, 2016

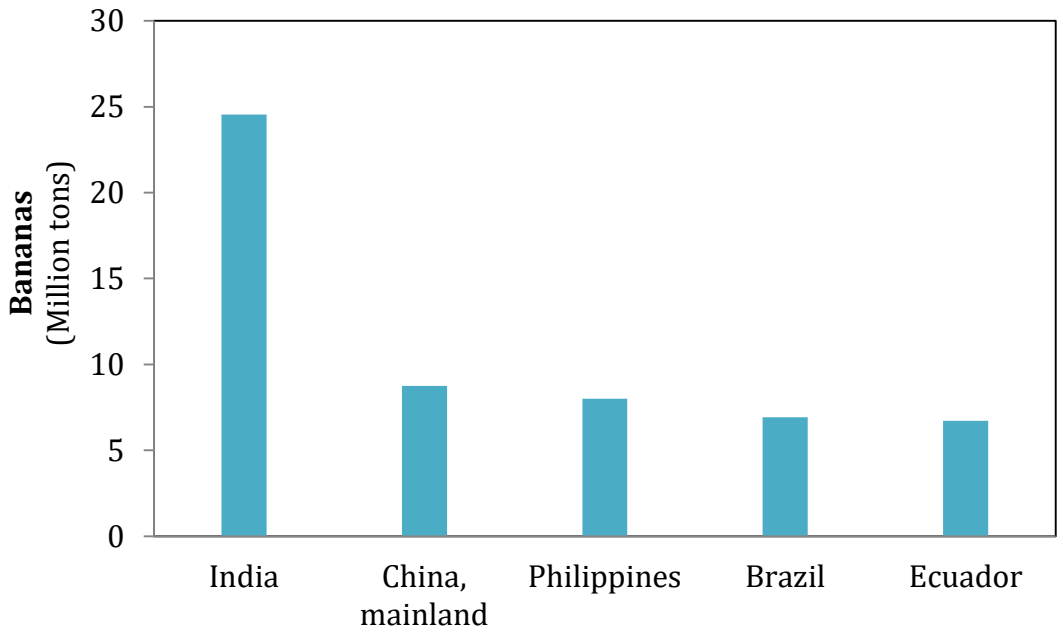


Figure 1.2.2 Top 5 banana producters in 2004-2014, Source: FAO, 2016

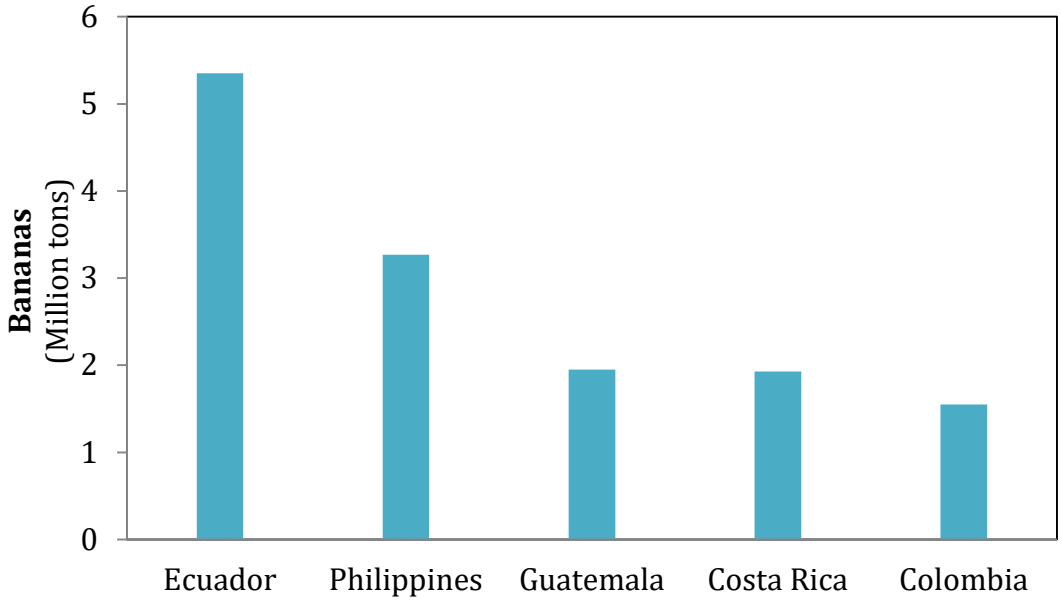


Figure 1.2.3 Top 5 banana exporters in year 2013, Source: FAO, 2016

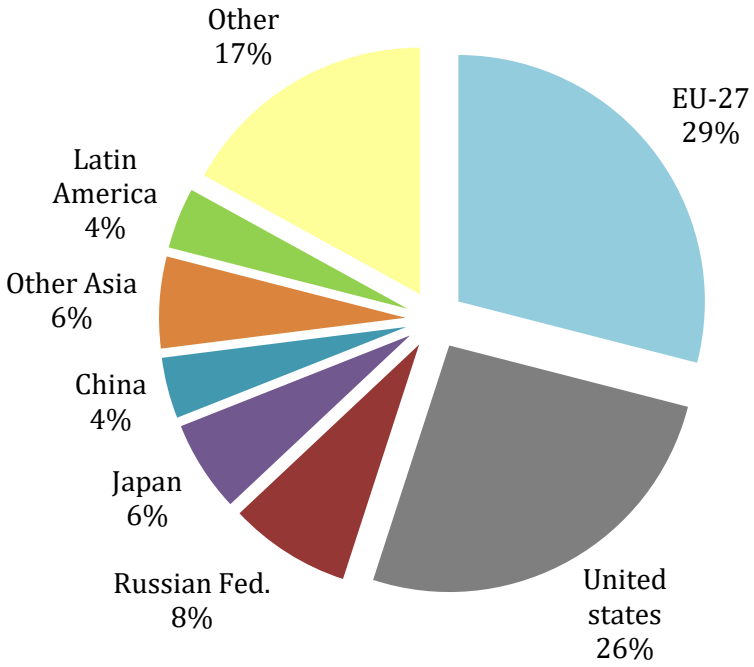


Figure 1.2.4 Countries share in global banana imports by market in year 2013,

Source: FAO, 2015

1.3 Postharvest physiology of bananas

Banana fruit is an important economic crop in the world known as excellent sources of vitamins, year-round selling available, various health full-fill nutrients and also medicinal properties (Kumar et al., 2012). The fruits constitute a cluster form is called a 'hand' with an individual fruit called a 'finger' (Paull and Duarte, 2011). According to color index as described in Figure 1.3.1., the banana stage can be divided into pre-climacteric (green color, No.1), climacteric (green with yellow, No. 2-5) and post-climacteric (yellow, No. 7-8) phase. After banana is being harvested at the stage of all green unripe-maturity (Momen et al., 1997), their

steady metabolic system activates during ripening leading to changes regarding appearances, flavor, and texture.

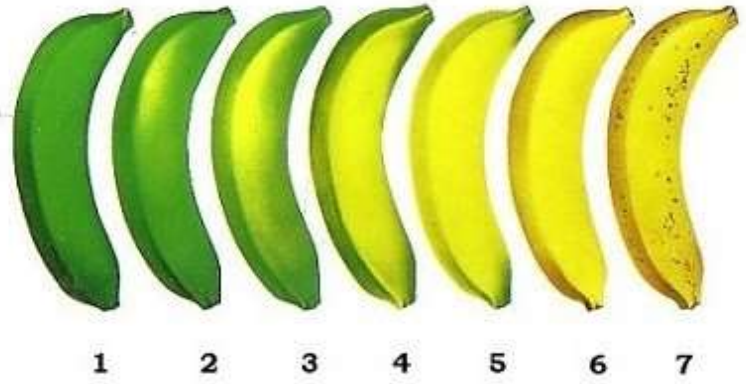


Figure 1.3.1. Color index of banana is determined by peel color where no. 1: green, 2: green trace of yellow, 3: more green than yellow, 4: more yellow than green, 5: green tip, 6: all yellow and 7: yellow-hatched with brown. Source: www.isopaninsulation.com, 2017

Pre-climacteric phase - banana that undergoes 75% maturity with some angularity are commercially harvested. At this stage, fruit contains 2.80 g/100g of starch and 1.26 g/100g of total sugar (Yap et al., 2017). The respiration releases small and steady rate of 20 mg CO2/ kg/ hour with no detectable ethylene production. The banana in this pre-climacteric period is sensitive to change in maturity which storage temperature, exogenous ethylene and other factors influence the process of ripening (Marriott and Palmer, 1980). Thus during transportation, cooling storage systems are carefully monitored to control the temperature at 13°C to preserve the quality and prevent chilling injury at a lower temperature. As regarded with the physiological age of banana, the pre-climacteric

stage has less susceptible to wound anthracnose than over-ripe stage (No.7) (Chillet et al., 2007). In addition to natural ripening, eternal ethylene is used to hasten the process that even 0.1 ppm, a minimum level of ethylene is sufficient for the ripening (Watada, 1986). Exogenous ethylene from 1 ppm to 1000 ppm applied to this stage is to initiate and uniform ripening of banana for consumptions.

Climacteric phase – this phase called as 'turning point' in short period depending on storage condition. Specifically at peel index no. 2 and 3, ethylene production accompanying with respiration rate rises immediately in the regular position to the highest level. This metabolic change is known to be driven by autocatalytic ethylene produced by itself or exogenous ethylene. The increase of respiration is more rapidly induced by external ethylene (Gane, 1937). By mode of action of ethylene, the ripening process will be subsequently changed, for instance, the modifications of the cell wall, degradation of starch and chlorophyll (Alexander, 2002).

Post-climacteric phase – banana is consumable, peel color develops full yellow, pulp texture becomes soft, sugar content increases and volatiles release. In this process, respiration and ethylene production decrease while some ongoing enzyme activities are increasing. After the post-climacteric stage, quality of the fruit deteriorates to the point where it is not desirable for consumptions. According to previous researches, an optimum temperature for ripening of banana is 20°C. However, fruit ripened at this temperature has shorter shelf-life compared to other temperature (Ahmad et al., 2001).

1.4 Postharvest physiology of fruits

Fruits ripening

Ethylene is a gaseous plant hormone involved in the ripening process of many fruits. It leads changes in fruit physiology including postharvest quality losses (Barry et al., 2000). The regulation of ethylene can be broadly classified by the ripening characteristics of fruits into two groups: non-climacteric, ethylene independence-regulated and climacteric, autolytic ethylene-regulated (Lelièvre et al., 1997).

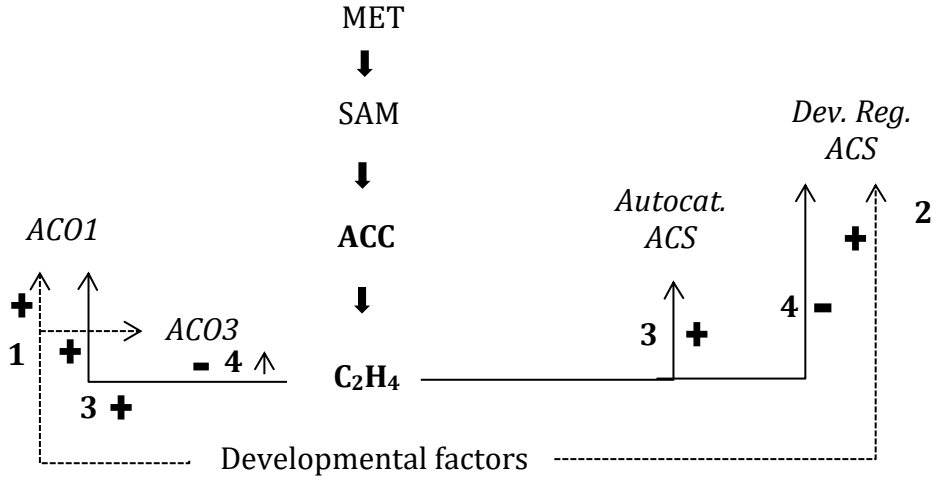


Figure 1.4.1 A model for the cascade of *ACS* and *ACO* gene expression during the transition to autocatalytic ethylene production in climacteric fruit. (1) The increase in *ACO1* and *ACO3* gene expression followed by (2) the increase in Dev. Reg. (Developmentally regulated) ACS activity would initiate a rise in ethylene production. This increase, in turn, would (3) induce autocatalytic *ACS* gene expression and stimulate *ACO1* and finally (4) down regulate Dev. Reg. *ACS* and *ACO3*. (Redrawn from Lelièvre et al., 1997)

As shown is the figure 1.4.1, MET is a precursor which requires intermediates ACC for producing ethylene. Multiple *ACO* and *ACS* genes are developmental factors involved in ethylene production. Fruits develop color, aroma, sugar and softer texture during the ripening process. Changes of the biochemical and physiological structure of fruits also occur. In this period, full ripen fruits have the most attractive appearance for customers. However, ripened fruits are highly susceptible to diseases and insects, which would lower the market value of the product.

Fruit softening

Softening is a remarkable physiological event during the development of fruit ripening. Climacteric fruit undergoes rapid softening during the maturation. Changing of postharvest quality during ripening in climacteric fruit is prominently consists of loosening of texture via disassembles of the cell wall by cell wall modifying proteins and enzymes (Brummel and Harpster, 2001).

The primary cell wall of plant cell composed of 25% cellulose, 35% pectins, and 25% hemicellulose (Baldwin and Pressey, 1989). Pectins are major components in middle lamella. Endo-polygalacturonase (PG) EC 3.2.1.15, pectate lyase (PL) EC 4.2.2.2, pectin methyl esterase (PME) EC 3.1.1.11 and rhamnogalacturonase are major pectin degrading enzymes, which are involved in tissue softening of fruit (Prasanna et al., 2007). PME associated with PG modifies cell wall structure leading to the breakdown of pectin chains (Figure 1.4.2 A). From the study of Marín-Rodriguez et al. (2002), it suggested that PL enzyme has a significant role in fruit

softening by β-elimination reaction (Figure 1.4.2 B). The Systematic name of PME, PG and PL from the database of IUBMB enzyme nomenclature (2017) are pectin pectylhydrolase, (1→4)-α-D-galacturonan glycanohydrolase and (1→4)- α -D-galacturonan lyase, respectively.

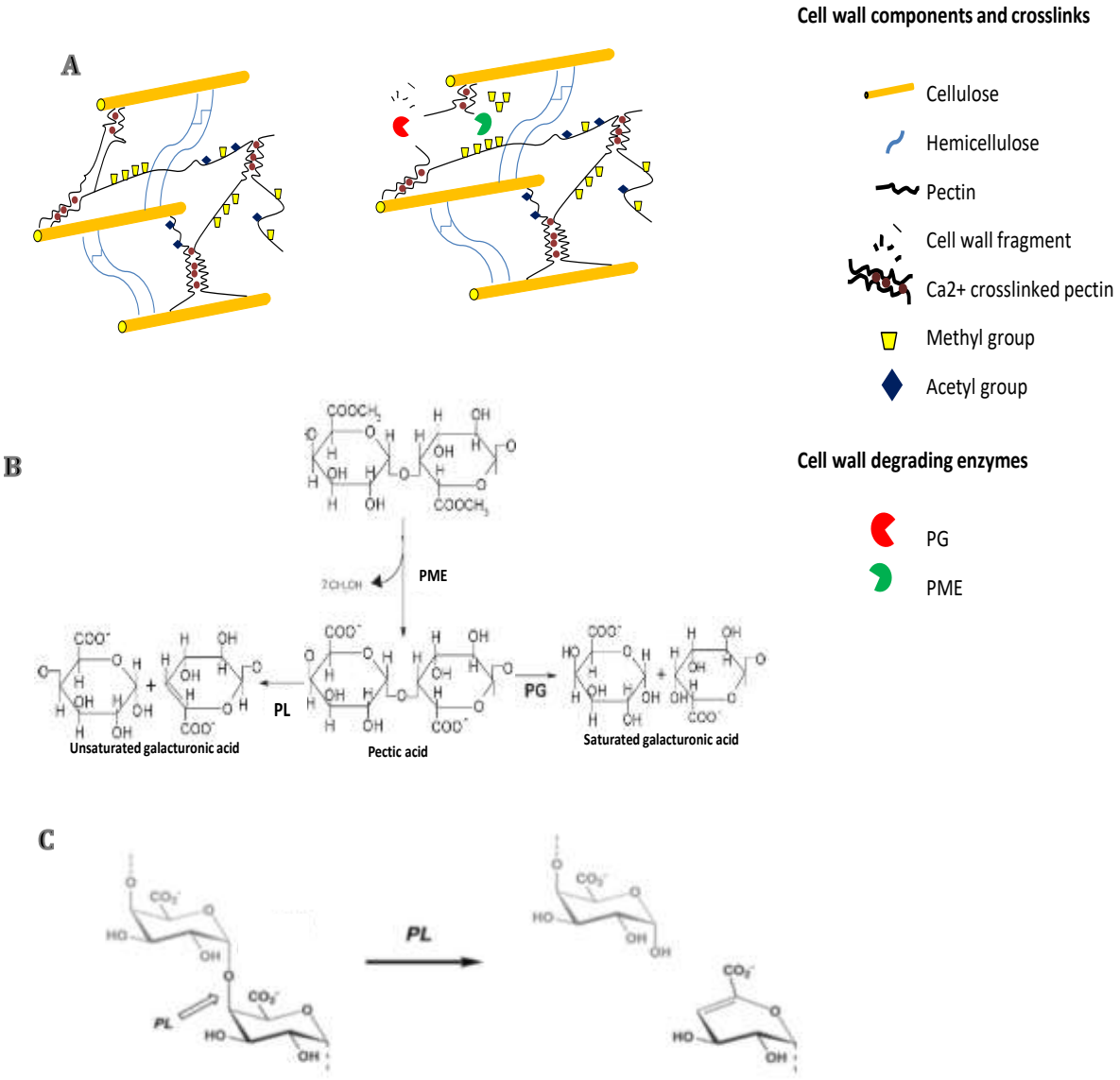


Figure 1.4.2 (A) Cell wall structure which is de-structured by cell wall degrading enzymes: PGs assisted by PMEs degrade cell wall polymers (Partially

adapted from Bellincampi et al., 2014). (B) Mode of action of pectinase: methyl group is removed by PME followed by hydrolysis of PG and (C) catalyzed the cleavage by β-elimination reaction of PL (Partially adapted from Combo et al., 2011 and Lombard et al., 2010). PME: Pectin methyl esterase, PG: Polygalacturonase, PL: Pectate lyase.

1.5 Postharvest heat treatment

The introduction of heat treatments for disinfestation of potato seeds began in 1882 by using hot water (Hara, 2013). Considering a potential of high-temperature treatment for removing undesired organisms from raw commodities, extreme temperatures were the first method of quarantine treatment, which was later temporarily replaced by inexpensive fumigants in the early 1950's (Mangan and Hallman, 1998). Later that year, however, it was discovered that the ethyl bromide used in the treatment was one of the carcinogens (Mangan and Hallman, 1998). Regardless of danger, the practice has still been used nowadays to satisfy the high demand for fruits. However, consumers concern the use of pesticides in respect of food safety, so their usages may not be preferable. Postharvest quality of horticulture crops is fast deteriorated with short shelf life. Particularly, when the pathogen attacks a part of the fresh tissue. Heat treatments are being used against fungal pathogens and physiological disorder to improve the postharvest quality of vegetable and fruits without fumigant application (Klein and Lurie, 1992). Additionally, heat treatments are the only method among various treatments that have the ability to reduce chilling injury (Mahajan et al., 2014).

Postharvest heat treatments consist of hot water dips or sprays, vapor heat, and hot air. The first two methods have been used for disinfestation (Lurie, 1998) while hot air has been utilized to study heat responses of commodities for promising treatments (Klein and Lurie, 1992). One of the disadvantages of heat treatments is the injury of plant tissue by high temperature. Thus, it is necessary to evaluate the optimum conditions by differential temperatures and durations for successful heat treatments. Postharvest ripening responses to temperature depend on cultivar, fruit size, and state of ripeness as well as heat transfer rate (Paull and Chen, 2000). Even through heat treatment is an efficient and sustainable postharvest disinfestation method for exporting of horticultural crops (Hara, 2013). However, the adoption of heat treatments by industry is not coinciding with the emphasized trend of researchers (Ferguson et al., 2000). Banana, as well as other fresh fruits, is inspected before its wild spread to importing countries. This step is concerned with quarantine treatment to prevent contaminants and non-existed pest that may harm and damage to industrial crops. Even though fumigation of chemicals could confirm undesired contaminants on fruits, are killed. The awareness of its side effect and residues of pesticide tend to be displayed unpleasant method for customers. Considering the difficulty of use of pesticides, non-chemical methods are engaging means to alternate a conventional practice.

1.6 The influence of heat treatment on postharvest fruit responses

Among other therapies, heat regulates postharvest fruit ripening by inhibiting a ripening, hormone, and ethylene (Klein and Lurie, 1992). Through an

extension of heat treatment on quality improvement and removal of contaminants in postharvest quality of fresh fruit, heat treatment is usually affecting the physiological changes and ripening process in particular. As mentioned in the previous section, the influence of heat treatment on fruit ripening depends on many factors (Paull and Chen, 2000). The factor researcher has less focused on is a profile of fruit growth condition, which is different in temperatures histories of the field and different postharvest heat treatments (Woolf and Ferguson, 2000). Plants itself are having the ability to cope with excessively high temperature referred as thermotolerance. Heat shock proteins (HSPs) are known to response to heat stress and to be synthesized and accumulated for a rapid heat (Wahid et al., 2007). The induction of heat shock responses occurs during the heating period. The constant expression of HSPs after heat treatment may play a significant role in the acquisition of thermotolerance (Bowen et al., 2002). The responses to heat stress of fruit ripening are categorized into normal stress cellular responses (< 40-42 °C) and exceeded cellular stress responses (> 45 °C) (Paull and Chen, 2000).

An interruption of ethylene production by heat treatments has been observed in many climacteric fruits. Avocado and Kiwi fruit failed to ripen normally coincided with the results of no ethylene production during ripening at high temperature (40°C) even the exogenous ethylene were applied after the treatment (Eaks, 1978, Antunes and Sfakiotakis, 2000). However, after removal of heat, the recovery of ethylene production was observed in mango fruit with delay at 38°C (Ketsa et al., 1999). By shortening of the time at a higher temperature (40°C to 50°C for 3 hours), ethylene production in Bayberry decreased resulting in delayed

ripening (Luo et al., 2009). The inhibition of ethylene production in heat treated fruits was found with suppression of both ACS and ACO activities (Ketsa et al., 1999), which are the key enzymes involved in ethylene synthesis required for autocatalytic ethylene production (Figure 1.4.1) (Lelièvre et al., 1997). Nevertheless, the influence of heat on fruit ripening is depending on differences in physiological characteristics as well as exposure time. For instance, hot water treatment caused heat injury in Hujin peach but not in Baihua peach, and also hot moist air at the same temperature as hot water treatment did not cause damage in Hujin peach (Zhou et al., 2002).

Cell wall degrading enzymes and ethylene production are mostly disrupted by heat (Paull and Chen, 2000). The inhibitory effects of postharvest heat treatments on cell wall degradation have been reported in pre-storage apples at the temperature of 38 °C for four days (Shalom et al., 1993). Decreasing of the rate of softening was observed in mango fruit treated with forced hot air at 43 °C for 220 min (Ornelas-Paz and Yahia, 2014). The action of PG causes drastic changes in pectin structure during fruit ripening (Fischer and Bennett, 1991). The suppressions of PG activity by heat treatments found in Chinese bayberry (Luo et al., 2009), strawberry (Vicente et al., 2005) and papaya (Chan et al., 1981). However, the reduction of PG activity did not coincide with the loss of firmness in mango fruit which was exposed to heat at 38°C for 3 hours (Ketsa et al., 1988). These results would indicate ß-galactosidase is a major enzyme involved in the softening progress of mango.

To develop technology for postharvest heat treatments for insecticidal treatment with imported banana fruits, Nakamura et al. conducted a first experiment in 2008 to control mealy bug on the surface of the banana. The authors observed that insect mortality decreased to zero at the condition of hot air at the temperature of 50°C for 10 -20 min. Consequently, in 2011 hyper vapor heat treatment was developed by the same authors. In the report, it suggested that at the temperature of 50°C for 10 min completely controlled the insect with no visible damage on banana's peel after removal of heat (Nakamura et al., 2011). However, in 2012, Nakamura et al. found out that at the thermal condition of vapor heat at 50°C for 10 min had an effect on ripening and pulp softening of banana. The authors reported that ripening and softening delayed. However, details of the consequences of heat treatment on softening process of the pulp have not been clarified.

Objectives

The aims of this thesis are;

I: To investigate the effects of hot air treatment on softening of banana, specifically the pulp tissue and cell wall degrading enzymes activities during storage with and without ethylene treatment.

II: To examine the effects of hot air treatment on expressions of genes related to ripening and softening of banana.

CHAPTER II

Effects of hot air treatment on cell wall degrading enzymes on pulp softening in banana and its ripening with ethylene treatment

2.1 Introduction

The use of heat treatment in bananas as a phytosanitary intervention in lieu of methyl bromide presents a high potential. In 1982, Armstrong revealed that hot water effectively controlled three fruit fly species at 50 °C for 15 min treatment in banana fruits. Nakamura et al. (2008) conducted the preliminary studies that mealybug is effectively controlled by using of hot air treatment at 50°C for 10-20 min. In 2011, Nakamura et al. developed vapor heat treatment in order to control mealybug and the vapor heat treatment at 50°C for 10 min resulted in 100% mortality of mealybug. The condition of this treatment was proved to pose no detrimental effect on fruit quality and shelf-life (Nakamura et al., 2012). However, the effect of the heat treatment on postharvest quality of banana has not been clearified, particulary softening of the pulp.

Banana pulp softens rapidly in a climacteric ripening phase. A key process in tissue softening of various fruit appears to be degradation of pectin (Prasanna et al., 2007), a major component of primary cell wall and the most soluble wall polysaccharides, composed of (1-4) α-D-galacturonic acids (Rose, 2003). Several major cell wall-degrading enzymes are involved in the softening. One is PME, which hydrolyses the methyl esters in pectin. This enables subsequent hydrolysis of the (1-4) α-D-galacturonan backbones by PG (Fischer and Bennett, 1991; Rose, 2003).

Another enzyme that plays an important role in fruit softening is pectate lyase (PL) (Marin-Rodriguez et al., 2002), which also catalyzes cleavage of (1-4) α-D-galacturonan backbones, but by a β–elimination (Fischer and Bennett, 1991). Loss of the integrity and structure of pectin via activities of these enzymes increases its solubility, and decreases in water-soluble pectin contents are strongly associated with softening during ripening (Gwanpua et al., 2014).

In general, ethylene has been used as a commercial practice for uniform ripening of banana. Hence, we assessed hot air treatment of 50°C for 10 min followed by ethylene treatment to study the responses to the hot air treatment of softening and enzyme activities related to cell wall degradation; PG, PME, PL of pulp in banana with ethylene treatment.

2.2. Materials and Methods

Plant materials

The green mature bananas (*Musa acuminata* 'Dwarf Cavendish' subgroup) were purchased from a local market at Ibaraki prefecture, Japan. Bananas were transferred to the laboratory of pomology, University of Tsukuba and kept in a temperature-controlled incubator which had been set at 13.5 °C. Hands of banana were separated into an individual finger. The fingers were divided into two groups of control and heat treatment.

Hot air treatment

Sets of five fingers with a T-type thermocouple connected to a GL400 midi logger (Graphtec Corp., Japan), attaching to the peel surface were arranged on an

aluminum tray and placed in a central position in a CI-310 incubator (ADVANTEC, Japan). When the peel temperature reached 50°C, the fruits were held at that temperature for 10 min (Figure 2.3.1) which adapted from Nakamura et al. (2008), then cooled in water at room-temperature (RT) (20°C), air-dried at RT for 4 hours and allowed to ripen during storage at 20°C in an incubator. After that, all fruits were subjected to ethylene 1000 ppm for 8 hours. Control bananas were treated identically except that they were incubated at room temperature when the heated bananas were incubated at 50°C.

The samplings were performed at 0, 2, 6, 10, 15 and 20 days after heat treatment. After firmness measurement, the pulp was cut into small pieces, frozen with liquid nitrogen and immediately kept at -80°C. For non-destructive measurements, color of peel was monitored daily.

Peel color

A portable colorimeter (Model CR-400/410, Konica Minolta Inc., Japan) was used to measure Hue angle (H°) of banana peel. The machine calibrated so that three different values were obtained from each side of the banana. An average Hue angle of each banana then calculated.

Pulp firmness

A FRT-50N food rheology tester (Imada Co.Ltd., Japan) equipped with a 2 mm diameter cylindrical probe connected to a 100 mm probe shaft was used to measure the firmness of the bananas' pulp. To prepare samples, each finger was divided into three equal parts using a sharp knife and one side was peeled. A cut part was placed

on the tester's platform, the bar penetrated 10 mm into the pulp at 5 mm sec^{-1} and the maximum penetration force was recorded.

Enzyme analysis

Enzyme preparation

The extract for measuring of enzyme activities was prepared by the method of Pathak and Sanwal, 1998 with a slight modification. Approximately 5 grams of pulp tissue was homogenized with an extraction buffer (0.02 M Na-Pi buffer, pH 7, neutralised 0.02 M EDTA, 1% Triton X-100, 0.02M cysteine-HCl, and 1 mM PMSF) followed by the protein precipitation step with $(NH_4)_2SO_4$. Subsequently, dialysis was proceeded to remove excess salt against the same extraction buffer. Clear supernatants were collected by centrifugation and used for further analysis.

PG assay

The assay was performed according to Pathak and Sanwal (1998) with a slight modification. To start the reaction, 0.3 ml prepared supernatant was mixed with 0.3 ml of 0.2 M HOAc buffer (pH 4.5), 0.3 ml of 1% Polygalacturonic acid (Sigma-Andrich) and 0.1 ml distilled water, respectively. The 1-ml total volume was mixed thoroughly using a vortex and then incubated at 37°C, 60 min. A reducing sugars released by the reaction were measured with DNS solution (Miller, 1959) consisting of 1% of 3, 5-dinitrosalicylic acid, 1% of NaOH and 0.05% of Na_2SO_4. After the incubation, 0.5 ml of DNS solution was added to the reaction mixture and boiled for 10 minutes. Following the heating step, 1 ml of 40% potassium sodium tartrate was added to the mixture immediately to stabilize color prior to cooling at room

condition. Control tubes received a substrate just before heating. The absorbance of the mixtures was measured at 540 nm using spectrophotometer (Jasco V-550, JASCO Corporation, Tokyo, Japan). The standard curve was made with D-galacturonic acid. One unit of PG was defined by releasing 1 nmol D-galacturonic acid in 1 min at 37°C.

PL assay

The assay was described by Payasi and Sanwal (2003) using TBA method of Waravderker and Saslaw (1959). Five percent (w/v) of polygalacturonic acid was prepared as for the enzyme substrate which was dissolved in 0.05 M Tris-HCl buffer, pH 8.5. The reaction mixture consisted of 0.45 ml of Tris-buffer (pH 8.5), 0.15 ml of 0.01 M $CaCl_2$, 0.20 ml of polygalacturonic acid solution, 0.20 ml of prepared supernatant and distilled water, respectively. The solutions were then incubated at 37°C for 7 hours. To terminate the reaction, 75 µl of 9% $ZnSO_4.7H_2O$ and 75 µl of 0.5 M NaOH were applied to the solution. The forming of white jelly-like pellets were removed by centrifugation at 1,500 x g for 10 min. After collecting of the supernatant, 0.2 ml was collected and mixed with 0.25 ml of 0.025 N periodic acid (preparing in 0.125 N H_2SO_4). The solution was then placed at ambient condition for 40 min which is optimal oxidation period for sugar bearing a trans grouping (Waravderker and Saslaw, 1959). Next, 0.5 ml of 2% (w/v) $NaAsO_2$ in 0.5 N HCl was continuously added and allowed to stand for 2 min. The solution (pH 2.0) was finally mixed with 2 ml of 3% (w/v) 2-thiobarbituric acid followed by boiling for 10 min. The absorbance of cooled solutions was measured at 548 nm. Non-incubated tubes

were used as control. One unit of PL activity was determined as an increase of 0.01 in absorbance against the control under assay condition.

PME assay

Prepared enzyme solutions required for all assay were adjusted to a pH 7.5 which consisted of solutions as follows: 0.5% (w/v) pectin substrate, 0.015% bromothymol blue in 3 mM KH_2PO_4-KH_2PO_4 buffer, 3 mM KH_2PO_4-KH_2PO_4 buffer and distilled water. Prior to adding of an extracted supernatant, 2 ml of pectin mixed together with 0.15 ml of bromothymol blue were incubated at 60°C for 2 min and then cooled to room temperature. Subsequently, 0.1 ml of extracted supernatant was added and immediately stirred. After 5 min, absorbance of the reading at 620 nm was measured. After addition of extracted supernatant, 1 min incubation tubes were used as control. One Unit of PME was liberated 1 μmol D-galacturonic acid in 1 min under enzyme assay.

Protein content

RC DC™ Protein kit (Bio-Rad Laboratories, Inc., Tokyo, Japan) was used to assay protein quantities based on Lowry method (Lowry et al., 1951). Calibrate curves were made with bovine serum albumin (BSA) serum.

2.3 Results and Discussion

Peel color

The yellow color develops in the peel of banana fruit is a physiological change during ripening (Figure 2.3.1) which is initiated by endogenous ethylene or exogenous ethylene. Hue angle indicates the development of peel color of bananas.

Hue angle of yellow peel was less than 97° and that of green color indicates greater than 104° (Yang et al., 2011). Ripening of banana fruit was accelerated by ethylene application in this experiment, thus hue angle in the peel of control dramatically decreased throughout the storage. Increases in hue angle values of banana peel were decelerated by hot air treatment relative to control banana (Figure 2.3.3). A decrease in hue angle of control banana on day 4 indicated that peel color had developed yellow color during storage (Figure 2.3.3). Meanwhile, the value of hot air treated banana remained significantly higher than that of control from day 2 to day 20 reflecting that peel de-greening was retarded.

From the results, it was suggested that hot air treatment delayed the degreening of banana peel. In banana, it was reported that, the degreening is inhibited by at temperature above 24°C which may be related to delay of breakdown of chlrophyll b (Blackbourn et al., 1990). Yang et al. (2009) reported that reduction of Mg-dechelatase activity may contribute to the repression of chlorophyll degradation in banana peel and also lead to uneven degreening. It was obvious that high temperature influenced the external appearance of banana peel and caused heat injury at 55°C (Mansour et al., 2006). Therefore, hot air treatment may effect on chlorophyll degradation in banana peel by inhibition of the degrading process or the ripening metabolism. This inhibition of de-greening by hot air assumed in coordination with ripening initiating interruption.

Pulp firmness and activities of cell wall degrading enzyme

A change of firmness of the banana pulp is indicated in Figure 2.3.4. The hot air treatment at temperature of 50°C evidently maintained of banana pulp firmness. After the treatment for 2 day, pulp firmness of control banana significantly decreased whereas, the firmness in hot air treated banana was postponed and did not decrease until day 6 after storage.

It was similar to the result of Varit and Songsin (2011) that softening of banana treated with hot water treatment was delayed for 3 days. Exposure of fruits with vapor heat at 48°C for 5 and 10 min. resulted in firmer avocado fruit (Bard and Kaiser, 1996). Also, in strawberry fruit, the flesh exposed to postharvest heat treatment (45°C for 15 min) was firmer than untreated one (Garcia et al., 1995). It was reported that heat treatment (45°C, 3 h air oven) effectively suppressed softening in strawberry (Martínez and Civello, 2008).The result suggested that delay of pulp softening by hot air treatment may be related to low activities of cell wall degrading enzymes.

PME, PG and PL activities after the treatment were demonstrated in Figure 2.3.5. The increase in PG activity of control banana pulp was correlated with decreases of pulp firmness. Among those enzymes, PG in the pulp of hot air treated banana exhibited lower activities than control at day 0 and 2 after the treatment. However, during decreasing of pulp firmness in banana exposed to hot air, activities of all three cell wall degrading enzymes increased slowly compared to the activities of control. It was reported that softening of apple was retarded by pre-storage heat treatment (38°C, 4 days) (Klein et al., 1990, Shalom et al., 1996). The authors discussed that the heat treatment inhibited uronic acid degradation in cell wall and

the solubilisation of the carbonate soluble fraction and resulted in firmness retention (Shalom et al., 1996). In our study, the increase of PME activity was inhibited in hot air treated banana. On the contrary, PME activity was increased by heat treatment in apple and strawberry (Shalom et al., 1996, Vicente et al., 2005). This may explain by the differences between softening characteristic of banana and mentioned fruit. In papaya, hot water treatment (46°C for 65 and 90 min.) caused delayed softening which is correlated with a decrease of PG activity (Chan et al., 1981). It was reported that hot air treatment suppressed the increase of PG and PME activity in bayberrry (Luo et al., 2009). Nevertheless, there were no significant difference between PL activities of hot air treated banana and untreated group. These results suggested that hot air retarded softening in banana pulp by the suppression of activities of PME and PG enzymes.

Conclusion

In this chapter, banana was treated with hot air treatment followed by the treatment of ethylene. Our results indicated that hot air delayed de-greening of the peel in which hue angle remained higher than control banana. Softening developed progressively in the pulp of control bananas throughout storage whereas it was suppressed until day 10 after storage in hot air treated bananas. PG activities in hot air treated banana pulp were lower than that in control until day 20. The increases of PME activity and PL activity in hot air treated bananas were observed at day 15 and day 10, respectively. The results suggested that hot air delayed the ripening including softening of bananas. Softening of the banana pulp was delayed by hot air treatment may be regulated via the inhibition of PG, PME and PL activities.

Despite of the fact that hot air inhibited activities of cell wall degrading enzymes and ripening observed in our study. External ethylene, which has been used as a commercial practice for uniform ripening in banana, induced ripening. Since, ethylene induced these physiological changes. It seemed to make confusing to discuss on how hot air treatment effect on banana fruit pulp. Thus, to clarify the effect of hot air treatment and specify its effect on softening without exogenous ethylene, we conducted the experiment in the following chapter.

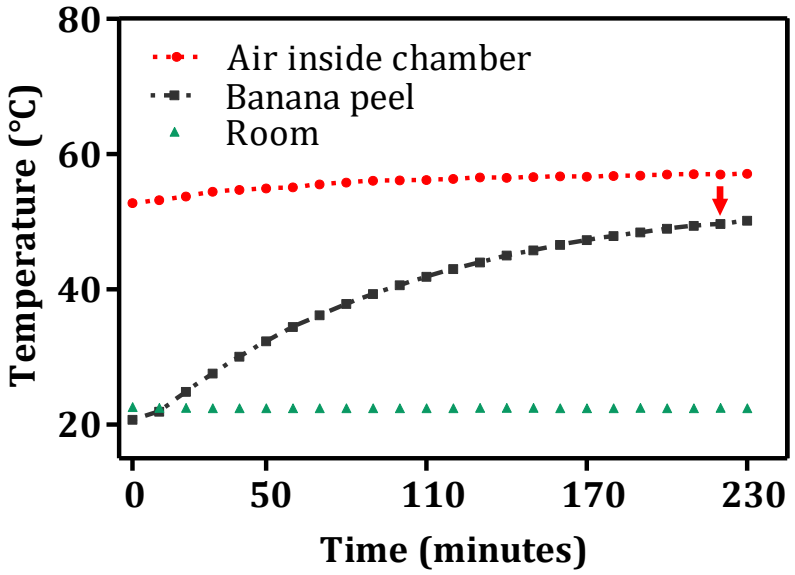


Figure 2.3.1 Time course of temperature changes during the hot air treatment. The surface temperature of mature green bananas reached 50°C at the time indicated by the arrow, and were then held at that temperature for 10 min. Data are means ± S.E. of three replications.

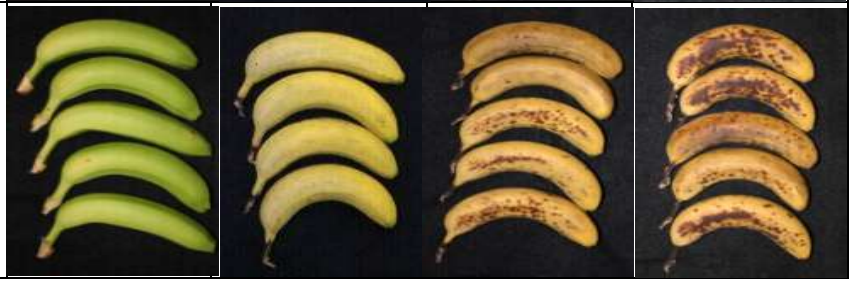
	Days after storage			
	0	**9**	**15**	**20**
Control				
Hot air				

Figure 2.3.2 Appearance of hot air treated (50 °C for 10 min) and untreated fruit (RT for 10 min). All fruits were treated with 1000 ppm ethylene for 8 hours and ripened at room temperature (20°C).

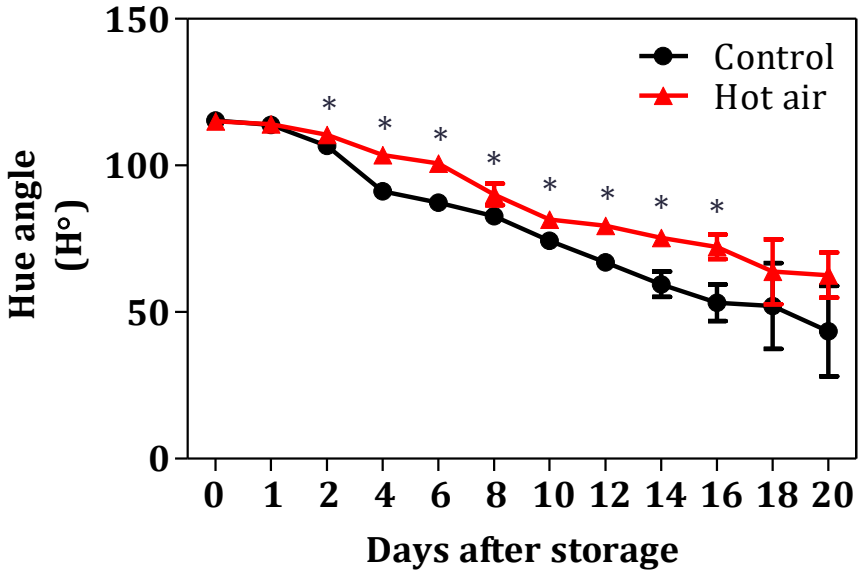


Figure 2.3.3 Changes of hue angle in the peel of hot air treated (50 °C for 10 min) and untreated fruit. All fruits were treated with 1000 ppm ethylene for 8 hours and ripened at room temperature (20°C). Data are means ± S.E. of three replications. An asterisk indicates significant difference at $P \leq 0.05$ by t-test.

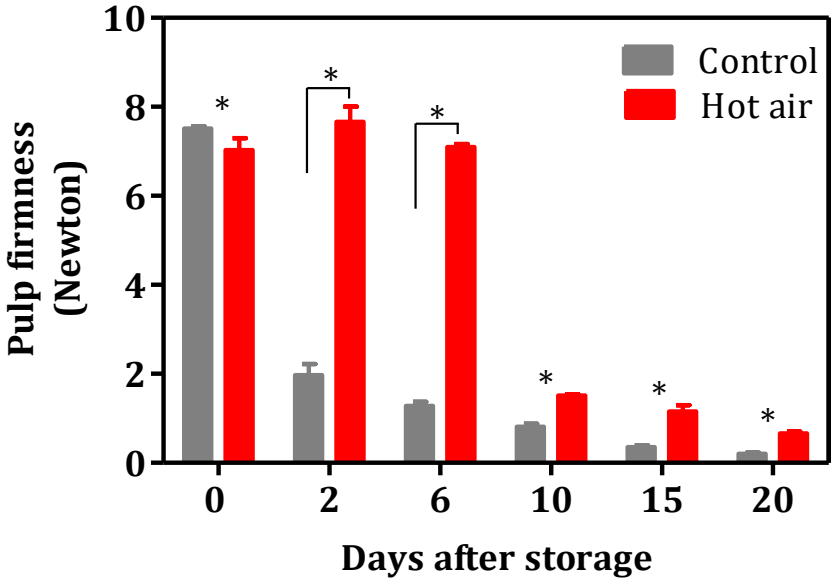


Figure 2.3.4 Pulp firmness of hot air treated (50 °C for 10 min) and untreated fruit. All fruits were treated with 1000 ppm ethylene for 8 hours and ripened at room temperature (20°C). Data are means ± S.E. of three replications. An asterisk indicates significant difference at $P \leq 0.05$ by t-test.

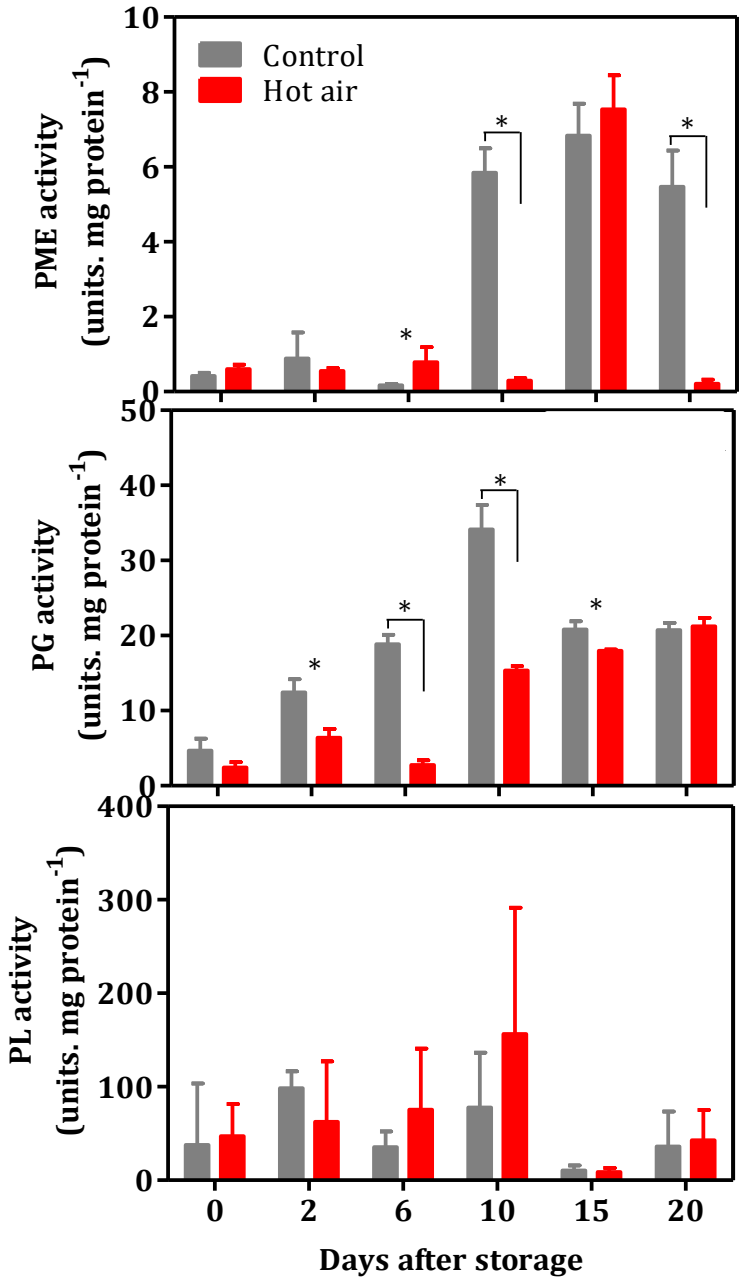


Figure 2.3.5 Activities of PME, PG and PL enzyme in the pulp of hot air treated (50 °C for 10 min) and untreated fruit. All fruits were treated with 1000 ppm ethylene for 8 hours and ripened at room temperature (20°C). Data are means ± S.E. of three replications. An asterisk indicates significant difference at $P \leq 0.05$ by t-test.

CHAPTER III

Effects of hot air treatment on cell wall degrading enzymes on pulp softening in banana and its ripening

3.1 Introduction

Softening is noticeble physical changes during fruit ripening of bananas with changes of peel color, sugar and starch content and flavor. Heat frequently disturbs cell wall degrading enzyme and ethylene biosynthesis (Paull and Chan, 2000). Besides various advantage of heat treatment as high as 50°C with banana fruits seems applicable, it may affect banana tissue in such ways. It is necessary to investigate the effects of the treatment on banana pulp tissues from the aspects of softening processes. In the previous reports, activities of cell wall degrading enzymes of PME, PG and PL which are responded to softening are increasing during ripening of banana (Hultin and Levine, 1965, Pathak and Sanwal, 1998, Payasi and Sanwal, 2003). The activities of PME and PG were relatively high during pulp softening of banana (Kunasekaran et al., 2012). Additionally, it was reported that PL was abundant and plays importance role in wall disassembly during ripening in pulp (Medina-Suarez et al., 1997).

Heating can reportedly disrupt cell wall-degrading enzymes and ethylene production in fruits, but their responses depend on various factors, including the threshold temperature of their tissues for heat stress, which is typically ca. 45°C (Paull and Chen, 2000). The species involved may also be important. For example, Klein et al. (1990) found that incubating of apples at 38°C for 4 days resulted in

firmer pulp, but hot water treatment at 46°C resulted in softer mango pulp according to Jacobi et al. (1995). Moreover, the sensitivity of enzymes to heat treatment may depend on the ripeness of the tissues (Lazan et al., 1989).

In the previous chapter, we investigated the effects of hot air treatment on softening process using bananas ripened with ethylene as commercial bananas. Banana is a climacteric fruit and ripening is regulated by endogenous ethylene and exogenous ethylene. In the hot air treated banana with exogenous ethylene, effects of hot air treatment itself was not clear. Thus, we conducted similar experiment to the experiment in the previous chapter without ethylene treatment. The aim of the study presented here was to elucidate effects of hot air treatment on banana fruit quality and cell wall-degrading enzyme activities during storage without exogenous ethylene treatment.

3.2 Materials and Methods

Plants materials

Green mature bananas (Musa acuminate 'Dwarf Cavendish' subgroup) were purchased from a local market in Ibaraki prefecture, Japan. They were transferred to the Pomology laboratory, University of Tsukuba, and kept in a temperature-controlled incubator set at 13.5°C. Fresh materials were prepared by separating each banana cluster into individual fingers, which were divided into two groups, one assigned to a heat treatment (described below) and the other to a control treatment.

Hot air treatment

Sets of five fingers with a T-type thermocouple, connected to a GL400 midi logger, attached to the peel surface were arranged on an aluminum tray and placed in a central position in a CI-310 incubator (ADVANTEC, Japan). When the peel temperature reached 50°C, the fruits were held at that temperature for 10 min which adapted from Nakamura et al. (2008), then cooled in water at room-temperature (20°C), air-dried at RT for 4 hours and allowed to ripen during storage at 20°C in an incubator. Control bananas were treated identically except that they were incubated at room temperature when the heated bananas were incubated at 50°C.

After 1, 3, 9 and 15 days of storage three fingers of hot air-treated bananas and three controls were subjected to firmness measurements, and then the fresh pulp was cut into small pieces, frozen with liquid nitrogen and immediately kept at -80°C until further analysis, as described below. Three replications of hot air-treated bananas were also subjected to firmness measurement, cutting and freezing after 33 and 36 days of storage. Sampling for non-destructive measurements such as respiration rate, ethylene production, weight loss and hue angle, also described below, was done daily.

Peel color

The measurement was previously described in the materials and methods of chapter 2.

Pulp firmness

The measurement was previously described in the materials and methods of chapter 2.

Weight loss

The weight of each finger was recorded daily using a digital scale and the resulting data were used to calculate the percentage weight loss at each day since the start of storage.

Respiration and ethylene production of fruits

To collect gasses released from the bananas they were incubated in a 1-liter closed plastic container at 20°C for 1 hour. Then, 1 ml of the head space gas was taken and injected into a GC - 18A gas chromatograph (Shimadzu co. Japan) equipped with a total conductivity detector and a Porapak Q (Mesh 60/80) column (Shimadzu Co., Japan) to determine the CO_2 concentration. In addition, a 1 ml sample was used to measure the ethylene concentration in the headspace, using a GC - 8A gas chromatograph (Shimadzu co. Japan) equipped with a flame thermionic detector and a WG - 100 column (also supplied by Shimadzu Co.).

Enzyme analysis

Enzyme preparation

Extracts for measuring enzyme activities were prepared following Pathak and Sanwal (1998), with a slight modification. Samples (ca. 5 g) of pulp tissue were homogenized in a buffer containing 0.02 M Na-Pi buffer, 0.02 M EDTA, 1% Triton X-100, 0.02 M cysteine-HCl, and 1 mM PMSF (pH 7), then proteins were precipitated

with $(NH_4)_2SO_4$, dialyzed to remove excess salt and centrifuged as described by the cited authors. The supernatants were then used for the analyses described in the following sections.

PG assay

PG activity was assayed following Pathak and Sanwal (1998) with a slight modification. To start the reaction, 0.3 ml of the supernatant described above was mixed with 0.3 ml of 0.2 M acetic acid buffer (pH 4.5), 0.3 ml of 1% polygalacturonic acid (Sigma-Andrich) and 0.1 ml distilled water. The resulting preparation was thoroughly vortex-mixed then incubated at 37°C for 60 min. Then, 0.5 ml of DNS solution (Miller, 1959), containing 1% of 3, 5-dinitrosalicylic acid, 1% NaOH and 0.05% Na_2SO_4 was added. The mixture was boiled for 10 min, then 1 ml of 40% potassium sodium tartrate was immediately added to stabilize color (formed from the reaction between DNS and reducing sugars) prior to cooling at room temperature.

Control tubes were not incubated after receiving the supernatant. The absorbance of the mixtures at 540 nm was measured using a V-550 spectrophotometer (JASCO Corporation, Tokyo, Japan). A standard curve was obtained using D-galacturonic acid, and one unit of PG was defined as the amount required to release 1 nmol D-galacturonic acid in 1 min at 37°C.

PL assay

PL activity was assayed using the 2-thiobarbituric acid (TBA) method of Waravdekar and Saslaw (1959) as described by Payasi and Sanwal (2004). A 5%

(w/v) solution of polygalacturonic acid in 0.05 M Tris-HCl buffer (pH 8.5) was prepared as the enzyme substrate. 0.20 ml of this solution was mixed with 0.45 ml of Tris-buffer (pH 8.5), 0.15 ml of 0.01 M $CaCl_2$, 0.20 ml of the supernatant described above and appropriate amount of distilled water.

The mixture was then incubated at 37°C for 7 hours. To terminate the reaction, 75 µl of 9% $ZnSO_4.7H_2O$ and 75 µl of 0.5 M NaOH were added. The white jelly-like pellets formed were removed by centrifugation at 1,500 x g for 10 min. A 0.2 ml portion of the supernatant was then mixed with 0.25 ml of 0.025 N periodic acid (prepared in 0.125 N H_2SO_4). The solution was subsequently incubated at room temperature for 40 min, the optimal oxidation period for sugars with *trans* groups (Waravdekar and Saslaw, 1959). Next, 0.5 ml of 2% (w/v) $NaAsO_2$ in 0.5 N HCl was gradually added and the mixture was allowed to stand for 2 min. The solution (pH 2.0) was finally mixed with 2 ml of 3% (w/v) TBA then boiled for 10 min. After cooling the absorbance at 548 nm of the solutions (and non-incubated counterparts as controls) was measured, and one unit of PL was defined as the amount required for a 0.01 increase in absorbance units relative to controls under these conditions.

PME assay

To assay PME activity, initially 0.5% (w/v) pectin substrate, 0.015% bromothymol blue in 3 mM KH_2PO_4-KH_2PO_4 buffer, and 3 mM KH_2PO_4-KH_2PO_4 buffer solutions, all adjusted to pH 7, were prepared. 2 ml of the pectin and 0.15 ml of the bromothymol blue solutions were mixed and incubated at 60°C for 2 min, then cooled to room temperature. Subsequently, 0.1 ml of the supernatant described

above was added, the mixture was immediately stirred and 5 min later the absorbance at 620 nm was measured. One min after addition of the supernatant incubation tubes were used as controls and one unit of PME was defined as the amount required to liberate 1 μmol D-galacturonic acid in 1 min under these conditions.

Protein content

An RC DC™ Protein kit (Bio-Rad Laboratories, Inc., Tokyo, Japan) was used to determine amounts of proteins in the samples, following Lowry et al. (1951). Calibration curves were prepared using serum BSA.

Statistical analysis

Statistical design of experiment, heat treatment and control was considered as independent (unpaired) samples. Consequently, T-test with Tukey's test, $P \leq 0.05$ was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3.3 Results and Discussion

Peel color

Yellow coloration rapidly developed in the peel of control banana during storage as the Hue angle sharply decreased from 113.74° to 89.28° between day 6 and day 9, then further declined to 85.11° by day 15 (Figure 3.3.1A). The Hue angle of the peel of heat-treated bananas changed more slowly and remained higher than that of controls from day 9 to day 20. Considering the ripening stage of banana fruit,

storage of control group was ended on 15 days while heat treated group was delayed until day 36.

It is known that storage at temperatures above ca. 24°C retard loss of greenness and changes in Hue angle of banana peel (Kajuna et al., 1998). This is due to inhibition of chlorophyll degradation (Seymour et al., 1987; Thomas and Janave, 1992; Drury et al., 1999), which may involve inhibition of Mg-dechelatase activity (Yang et al., 2009). Our results show that hot air treatment can inhibit chlorophyll degradation, resulting in maintenance of green banana peel for up to 20 days.

Weight loss

All bananas dramatically lost their weight during storage at 20°C (Figure 3.3.1B), and the losses of hot air-treated and control bananas were similar until day 15, after which storage of controls ended and treated bananas continued to lose weight until the end of the experiment at day 36.

Banana fruits inevitably lose weight during storage (Ahmad et al., 2001), due to losses of respiratory gases and water vapor, and hot water treatment reportedly accelerates the losses both temperature- and duration-dependently (Amin and Hossain, 2013). This conflict with our finding that weight losses of hot air-treated and control bananas were very similar. A possible explanation for the discrepancy in results of the two thermal treatments is that the hot water treatment applied by Amin and Hossain caused re-arrangements of epicuticular layers that accelerated weight losses. For example, López-Castañeda et al. (2010) found that heat treatment caused fissure-reducing rearrangement of the epicuticular wax layer of apples that

reduced water losses. However, further research on the structure of peel by effects of heat treatment is needed to elucidate the mechanisms involved.

Respiration and ethylene production of fruits

Respiratory CO_2 and ethylene production rates of control bananas strongly rose after storage for 5 days, and peaked at days 6 and 8, respectively (Figure 3.3.1C, 3.3.1D). In contrast, no ethylene production by the hot air-treated bananas was detected until day 29 after treatment and their respiration rate remained continuously low until day 28. A small peak in ethylene production (0.2 μl/ kg/ h) was observed at day 30, and the respiration rate gradually rose after day 28 in the hot air-treated bananas.

A sharp increase in ethylene followed by a climacteric peak of respiration is general characteristics of banana fruit ripening (Liu et al., 1999; Pathak et al., 2003), which demonstrates that initiation of fruit ripening leads to major physiological and chemical changes (Millerd, 1953). Our results show that exposure to hot air at 50°C for 10 min. can strongly retard the increases in respiration and ethylene production in stored bananas, and the metabolic changes associated with ripening. In addition, among those of heat treatment, the effects on ethylene production were strongest in accordance with findings by Antunes and Sfakiotakis (2000) using kiwifruits. Discussion of heat effects on ripening of fruits and vegetables has also focused heavily on ethylene (Lurie, 1998) as it promotes climacteric ripening (Saltveit, 1999), and it is strongly involved in heat stress responses (Larkindale et al., 2005). Thus, the ripening-retarding effects of high temperature we observed may be due to

suppression of enzymes activities of ethylene synthesis (Morgan and Drew, 1997), e.g. ACS and ACO (Ketsa et al., 1999).

Heat stress responses are mediated by heat shock proteins induced by heat stress transcriptional factors in plants (Kotak et al., 2007). Temperatures above 45°C, around the short-term thermal damage threshold may perturb induction of heat shock-mRNAs in fruit, and hence general repair responses to less severe heat shocks, which include (inter alia) increases in respiration and suppression of ethylene production rate (Paull and Chen, 2000), in accordance with our findings.

Pulp firmness

The firmness of the control bananas' pulp dramatically declined during storage (Figure 3.3.2A), dropping from 7.15 to 1.32 Newton, as measured by the rheology tester, by day 9 and 0.87 Newton by day 15. In contrast, the pulp of hot air-treated bananas lost very little firmness during the first 15 days of storage although it fell strongly between 15 and 33 days. Our other findings show that the hot air treatment strongly retarded other ripening-related changes. Other authors have shown that heat treatments can retard softening of bananas (Ummarat et al., 2011) and various other fruits (Vicente et al., 2005; Luo et al., 2009; Dotto et al., 2011; Ornelas-Paz and Yahia, 2014).

Shalom et al. (1993) suggested that increases in fruits' firmness came from the loss of neutral sugar side chains during heating may lead to closer packing of pectin strands and thus hinder enzymatic cleavage during and after storage. Paull and Chen (2000) noted that heating often disrupts cell wall-degrading enzymes and

ethylene production, which may explain the delayed softening in banana pulp induced by the hot air treatment and (inter alia) observations that heat treatment can impair ethylene production and softening in papaya (An and Paull, 1990). In our experiment the small peak of ethylene detected at day 30 (Figure 2D) may have initiated ripening and the loss of pulp firmness in hot-air treated bananas we recorded at day 33.

PME activity

PME activity did not significantly change in either hot air-treated or control bananas up to day 9 (Figure 3.3.2B). Between days 9 and 15 it increased in both sets, but the activity was significantly stronger in controls. It also further increased after day 15 in the hot air-treated bananas. These findings are consistent with previous observations that PME activity in banana pulp increases during ripening (Hultin and Levine, 1965).

The findings also clearly show that the hot air treatment inhibited increases in PME activity. Suppression of PME activity by heat has been previously observed in Chinese bayberry (Luo et al., 2009) and banana (Mirshekari et al., 2015). Intriguingly, cold shock treatment can also reduce PME activity in banana fruits (Zhang et al., 2010). However, according to Brummell and Harpster (2001), transgenic suppression of PME activity does not affect the firmness of fruit during ripening in the absence of thermal shocks.

PG activity

PG plays an important role in banana softening, and increases in its activity coincide with reductions in pulp firmness during ripening (Pathak and Sanwal, 1998). We detected no PG activity at day 1 of storage in either hot air-treated or control bananas (Figure 3.3.2C). However, while PG activity progressively increased (and peaked at day 15) in the pulp of control bananas, no PG activity was detected in hot air-treated bananas until day 15 although it increased strongly thereafter until the end of the experiment at day 36. In both cases, as expected, the PG activity closely correlated with loss of pulp firmness. The results clearly suggest that ripening-associated increases in PG activity were strongly suppressed by the hot air treatment, confirming conclusions by Chan et al. (1981) that heat treatments depress PG activity, thereby delaying fruit softening in papaya. Heat-induced reductions in PG activity have also been previously observed in mango (Ketsa et al., 1988) and banana (Mirshekari et al., 2015).

These observations are consistent with several previous reports and conclusions regarding ripening and activities of PG and other pectin-metabolizing enzymes. Notably, Gwanpua et al. (2014) concluded that losses of neutral sugars, increases in pectin solubility and reductions in molar masses of water-soluble pectin are all associated with softening during apple ripening. In addition, Luo et al. (2009) found that suppression of PG and PME activity by heat resulted in delayed polymerization of pectic substances that can be dissolved by alkaline solutions containing chelators, and reduced increases in water–soluble pectic substances. As

PG activity is largely responsible for pectin depolymerization and increases

in its solubility (Brummell and Harper, 2001), as well as major changes in pectin structure (Fischer and Bennett, 1991) during ripening, the inhibitory effect of heat on PG activity may have contributed to the retarded softening in our hot air-treated bananas. However, a study with transgenic plants indicated that suppression of PG activity slightly reduces fruit softening (Brummell and Harpster, 2001). Furthermore, although we detected substantial PG activity at day 15 the pulp was still firm in our heat-treated bananas It is presumably that because the PG does not have sufficient time to influence firmness and/or activate other enzymes, softening were not perturbed by the hot air treatment.

PL activity

PL activity of control bananas continuously increased during their 15 days of storage (Figure 3.3.2D), as expected because it generally increases in pulp during ripening (Payasi and Sanwal, 2003; Lohani et al., 2004). In contrast, PL activity in hot air-treated bananas slowly increased and remained at low levels for 36 days. Thus, PL activity was strongly affected by the hot air treatment. Similarly, hot water treatment can reduce PL activity in banana peel (Amnuaysin et al., 2012) and pulp (Mirshekari et al., 2015).

These findings are consistent with reports which described that PL plays a significant role in wall disassembly during ripening, and is strongly up-regulated in ripened pulp (Medina-Suarez et al., 1997). In addition, Baldwin and Pressey (1989) found that both PG and PL solubilized uronic acids from washed cell wall fragments, but the PLs were most effective. Moreover, inhibition of PL could significantly

increase strawberry fruit firmness (Jiménez-Bermúdez et al., 2002). Thus, in our study the low activity of PL in heat-treated bananas relative to controls (detected at days 9 and 15) may have contributed to their weak losses of pulp firmness.

Conclusion

Our hot air treatment (50°C for 10 min) in the tested bananas without following ethylene treatment, clearly had inhibitory effects on their ethylene production and respiration, and delayed ripening processes including changes in peel color and pulp softening. Hot air treated-bananas greatly extended the shelf life up to 33 days. Softening is an important indicator of banana ripening that is caused by cell wall-degrading enzymes. Accordingly, increases in PG, PME and PL activities in banana pulp during storage were all reduced and retarded (relative to control levels) by the hot air treatment. Hence, in addition to other benefits of heat treatments they can retard softening of bananas. Our results may facilitate efforts both to understand effects of heat treatments on banana quality and improve postharvest treatments.

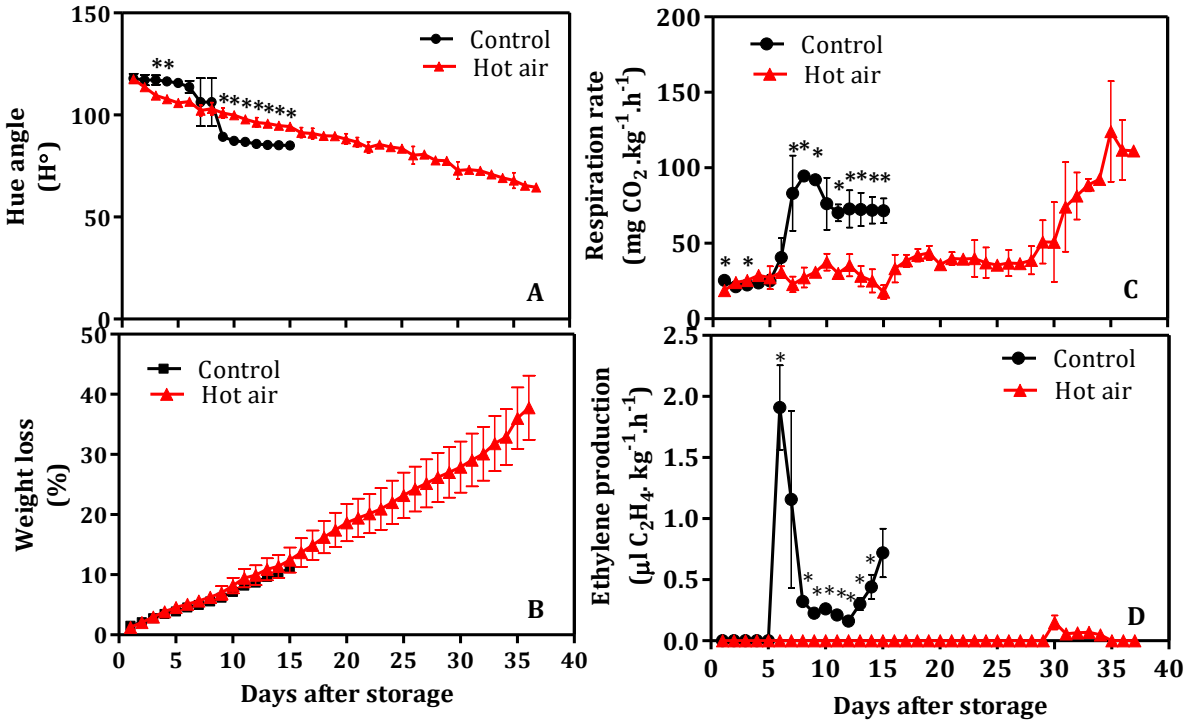


Figure 3.3.1 Changes in hue angle (A) of the peel, weight loss (B), respiration rate (C) and ethylene production (D) of bananas subjected to the hot air treatment (50°C, 10 min) or control treatment (room temperature, 10 min) then stored at 20°C. Data are means ± S.E. of three replications. Significant between-treatment differences ($P \leq 0.05$) are indicated by asterisks.

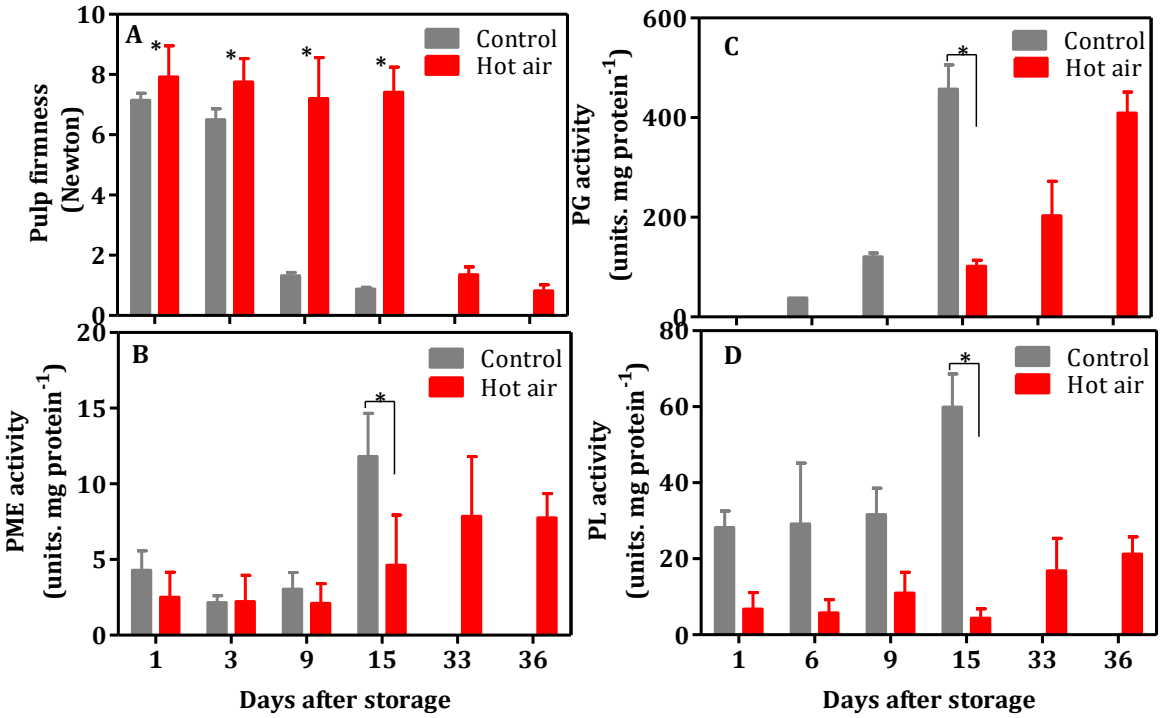


Figure 3.3.2 Changes in pulp firmness (A), and activities of PME (B), PG (C) and PL (D) enzymes in the pulp of bananas subjected to the hot air treatment (50°C, 10 min) or control treatment (room temperature, 10 min) then stored at 20°C. Data are means ± S.E. of three replications. Significant between-treatment differences ($P \leq 0.05$) are indicated by asterisks.

Chapter VI

Effects of hot air treatment on gene expressions involved in ethylene synthesis, softening and heat responses in banana pulp

4.1 Introduction

Banana is characterized as climacteric fruits. The ripening is initiated by ethylene, a gaseous hormone in plants which produce by the bio-synthetic pathway. This mechanism converts an intermediate S-adenosyl methionine (SAM) into ACC and thus, ACC to ethylene by ACC synthase (ACS) and ACO oxidase (ACO), respectively (Taiz et al., 2014). These two enzymes are encoded by multigene families and play an important role in ethylene production of banana fruit (Liu et al., 1999). In banana, it has been studied the characteristic of genes involved in ethylene synthesis and the reports demonstrated that the expression of gene encoding ACO in banana increased at the onset of ripening while *ACS* gene highly accumulated at the onset of the climacteric period (Huang et al., 1997, López-Gómez et al., 1997, Liu et al., 1999). Recently, at least nine members of *ACS* were observed in banana genome (*M. acuminata ACS* gene family) (Karmawan et al., 2009) and three different group of *ACO* gene clone (López-Gómez et al., 1997). According to the previous research, it has been reported that high temperature has effects on gene expressions involved in ethylene synthesis genes in broccoli, tomato and cut flowers (Lurie et al., 1996, Suzuki et al., 2005, Yangkhamman et al., 2007). Even though tomato is the climacteric modeling fruit (Nakatsuku et al., 1998, Barry et al., 2000), the effects of heat treatment on transcription of *ACS* and *ACO* genes in the plants may be able to explain the ethylene synthesis of banana. Moreover an ethylene-regulated pathway

in banana would differ from the pathways of other fruits (Pathak et al., 2003). In addition, the ripening process also varies between its pulp and peel tissue (Inaba et al., 2007). From our knowledges, the effects of hot air treatment on expressions of the genes involved in ethylene synthesis have not been reported.

The rigidity of texture caused by a high structural cell wall assembly containing cellulose, pectins, hemicelluloses, nonenzymic proteins, and lignin, a heterogeneous group of polysaccharides is the most abundant component of the most primary cell wall (Taiz et al., 2014). By hydrolysis action of cell wall degrading enzymes, PME removes a methyl group from esterified pectin facilitating PG to subsequent hydrolysis cleavage of galacturonide linkages and PL acts to cleavage of de-esterified pectin (Brummell and Harpster, 2001, Payasi et al., 2009). Then, cell turgor are loosened resulting in textural change. These enzymes mentioned above are encoded by multiple genes in banana (Inaba et al., 2007, Mbéguié-A-Mbéguié et al., 2009). Heat treatment was found that it suppressed *PG* gene expression in tomato kept at 34°C (Kagan-Zur et al., 1995). Moreover, peach fruit, which had been dipped in hot water at 60°C for a very short time (20s) also demonstrated a suppression of gene expressions of *PME*, *PG*, *PL* (Spadoni et al., 2014). In banana, Amnuaysin et al. (2012) described that hot water treatment reduced some cell wall degrading gene expression in the peel. Thus, the effect of hot air treatment on pulp softening in banana, which has never been studied before, will be examined at the transcription level. Moreover, it was reported that air oven used for heat treatment at 45°C for 3 hours. reduced the expression of *expansin* genes in strawberry (Dotto et al., 2011). Expansin is an extracellular protein that found abundantly in the pulp

of banana and has direct correlation with softening of banana (Trivedi and Nath, 2004) and other fruits (Brummell and Harpster, 2001). It is consisting of two major groups, α- and β-expansins (Taiz et al., 2014). It has been reported that α-expansin was encoded by four genes in banana (Asha et al., 2007). In the previous reports, *expansin* gene expression is regulated by ethylene and has cell wall disassembly function by local disruption of polysaccharide adhesion and enhancing the accessibility of non-covalently bound polymers during fruit ripening (Rose et al., 1997, Cosgrove, 2000a). Cosgrove (2000b) indicated that expansins are possibly facilitating an enzymatic attack. Thus, we also examine the effects of hot air treatment on transcription level of *expansin* genes which has never been well studied before in the pulp of banana.

The high temperature is one of abiotic stress in plants that causes metabolic disruptions. The stress leads the protein to dysfunction or destabilization and resulted in cell death. Heat shock proteins (HSPs) are induced to protect the cell against many stresses as self-defense function to cope with possible problems under unordinary conditions of the environments where normal protein conformation are re-established (Wang et al., 2004). Considering these facts, hot air treatments on harvested fruits may induce HSPs. These proteins would response to the change and the alteration of the gene expression (Timperio et al., 2008). Hot water treatment with rinsing at 62°C for the 20s was reported to activate various stress-related genes (Sapitnitskaya et al., 2006) that involved with heat shock proteins. Thus this could potentially display stress accumulation by heat treatments.

In the chapter III, the results illustrated that the hot air treatment inhibited the activities of cell wall degrading enzymes, ethylene production in banana. Hence, to study the effects of hot air treatment of various genes involved in ethylene synthesis, cell wall degradation and heat stress in the pulp of post-harvested banana, which has not been reported yet. In this chapter we maimed to study expression of genes encoding *ACO (MaACO1)*, *ACS (MaACS1*, *MaACS2)*, *PME*, *PG*, *PL*, Expansin (*Exp1*, *Exp2*) and *HSPs* (*HSP70*, *HSP90*) in the pulp of banana fruits with hot air treatment at 50°C for 10 min.

4.2 Plant materials and Methods

Plant materials

All materials for experiment in this chapter were same pulp tissues described in chapter II.

RNA extraction

Total RNA from pulp tissue of banana (samples used in this experiment was same as described in chapter III) was successfully isolated with hot borate/ Proteinase K method by the protocol of Mbéguié-A-Mbéguié et al. (2008), adapted with Wan and Wilkins (1994)'s procedure. The extraction process was indicated in Figure 4.2.1. For extraction, 1 gram of the frozen pulp was ground into powder and subsequently added to a 5 mL of heated mixed solution. The solution prepared by adding freshly 1% SDS, 1% SDA, 10 mM dithiothreitol and 0.5% Igepal to a solution composed of 200 mM BORAX, 2% PVP and 30 mM EGTA. The powder and solution thoroughly blended by a homogenizer for 2 min. After the incubation, 2.5 mg Proteinase K was added to the homogenous suspension and incubated at 42°C for

1.5 h with gentle shaking at 100 rpm. After that, KCl was added to final concentration of 160 mM and kept on ice for an hour. The solution was centrifuged at 12,000 *x* g for 20 min at 4°C.

The supernatant was then collected and LiCl was added to a final conc. of 2 M. and kept overnight at 4°C. The RNA-containing pellet was collected by centrifuging at 12,000 *x g* for 10 min at 4°C. The pellet was washed with 2 M LiCl and air dried for 10 min at room temperature. The pellet was dissolved in 1 mL Tris-HCl buffer, pH 7.0. Consequently, an equal volume of phenol: chloroform: (isoamyl alcohol) IAA (25: 24: 1) was used to extract the supernatant followed by chloroform: IAA (24: 1). An aqueous phase was selected and KOAc, pH 5.5 was added to a final conc. of 0.2 M then kept on ice for 30 min. The supernatant was then centrifuged at 12,000 *x* g for 10 min at 4°C and transferred into a new tube. Later, 99.5% cold ethanol was added to the supernatant and kept overnight at -20°C. The pellet was collected by centrifuging at 20,000 *x* g for 1 h. at - 10°C, then washed by 70% ethanol and air dried for 10 min at RT. Finally, the pellet was dissolved in 100 µL autoclaved DW and the purity and quantity of RNA were measured by using a spectrophotometer. The sample`s RNA preserved at -20°C for further analysis.

cDNA synthesis

A total 18 µl of the reaction mix consisted of 13µl of 2.5 µg RNA extracts, 4 µl reaction mix (SuperScript® VILO™ cDNA Synthesis Kit, Invitrogen) and 1 µl DNase I (Promega Co., USA) was to synthesize cDNA with PCR following the manufacturer's instructions. After the process had finished, 2 µl of the enzyme which also supplied

by SuperScript® VILO™ was added to the 18 µl of the reaction mix and synthesized the cDNA with PCR following the manufacturer's instructions. The cDNA was stored at –20°C for qPCR analysis.

Real-time quantitative PCR analysis

A reaction mixture of a total 20 µL volume for each cDNA sample contained 2 µL of diluted cDNA, 1 µL of primers (0.5 µL Forward: 0.5 µL Reverse = 10 µM), 10 µL Brillliant SYBR Green QPCR Master mix (Aligent, USA), 0.3 µL ROX (1:150 diluted reference dye) and 6.7 µL DW. The thermal profile recommended for Stratagene Mx3000P QPCR System (Aligent, USA) were initially set at 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. The melting curves were collected between 60°C to 95°C after 40 cycles and a single dissociation curve was determined the specificity of the amplification. After the analysis had completed, 10 µl of each PCR products was separated by 2% agarose gels in presence of ethidium bromide to ensure a single amplification product. The relative expression ratio of targeted genes was calculated by the efficiency (Ef) and Ct values of each cDNA sample normalized against Actin genes. Control banana at day 1 after storage was set as a calibrator nominating initial level of 1. Each primers and sources of genes used in this study are listed in Table 4.2.1.

Statistical analysis

Data analysis was designed as t-test at a significant level of 0.05 according to hot air treated banana and control during storage for 15 days.

4.3 Results

Ethylene biosynthesis genes

When banana fruits naturally ripened at 20°C (control), the expression of *MaACO1* in the pulp was sharply rose from day 1 to day 3 after storage and then reduced to 1.50 and 1.96 to their level at day 9 and 15, respectively (Figure 4.3.1). On the contrary, the expression of *MaACO1* in treated bananas significantly reduced at day 3. Then, there was no significant induction of *MaACO1* expression during day 1 to 15 after storage. Nevertheless, since the ripening of heat-treated bananas had commenced, the expression level of *MaACO1* was highly increased from day 33 to 36. Similar to the observations of *MaACO1*, the expression of *MaACS1* in the pulp of treated bananas was very low and less at day 33 and 36. The level of *MaACS1* expression in the control banana was continuously increased during storage from day 1 to 15. By extending a period of storage, heat-treated bananas exhibited extensive induction of the expression of *MaACO1* whose level raised from 295.82 to 1130 from day 33 to 36, respectively. Only gene expression of *MaACS2* was significantly induced at day 1 in the pulp by hot air in comparison to control.

Cell wall degrading genes

MaPME, MaPG, MaPL

Hot air inhibited the expression of all cell wall degrading related genes in the pulp of bananas (Figure 4.3.2). Hot air treated and untreated bananas did not show a significant difference in the relative level of *MaPME* expression at day 1 of storage. But after that, the expression level of *MaPME* in the pulp of control bananas

increased 2 times at day 3 compared to day 1, whereas in treated bananas, the expression was not clearly displayed. After 3 days of storage, the expression of *MaPME* in both of groups was low except bananas treated with hot air at day 15. The *MaPG* expression of control banana rose to the highest level at day 3 and gradually decreased afterward until day 15. On the other hand, the expression of *MaPG* was inhibited by hot air in the banana pulp during day 1 to day 9. The relative level had significantly lower than that of the control group, and by storing treated bananas until ripening stage at day 33 and 36, the expression level of *MaPG* induced at a level that was less than 1. *MaPL* had shown to activate and express after post-climacteric phase where the expression of *MaPL* progressed extensive induction at day 9 and 15 in the pulp of untreated sample. Even the expression of *MaPL* in bananas treated with hot air was not apparently detected until day 15, but at day 33 and 36, the expression was high where the level rose from 275 to 554, respectively.

Expansin genes

The expressions of expansin genes (*MaExp1*, *MaExp2*) in the pulp of control bananas were difficult to observe at day 1 and 3, but later both *MaExp1* and *MaExp2* were strongly induced at day 9 and 15 (Figure 4.3.3). By treating the fruit with hot air, *expansin* gene completely inhibited until day 15 then the induction of *expansin* genes was visible in treated-bananas at day 33, even thought *MaExp1* was decreased at day 36 whereas *MaExp2* disappeared.

Heat shock proteins

The treatment of hot air by heating at the temperature of 50°C for 10 min

significantly induced heat shock proteins (*HSP70*, *HSP90*) in the pulp of bananas for up to 1 day of storage (Figure 4.3.4). Both of *HSP70*, *90* expression levels were mainly induced in the pulp of hot air treated bananas after storage for 1 day compared to the control fruit. The level of *HSP90* was significantly high at 132 in bananas treated with hot air and 0.8 in untreated bananas. However, day 3, the expression of *HSP70* and *HSP90* in the pulp of treated-bananas rapidly decreased, and their level was lower than that of the control group. When the storage of heat-treated bananas was extended at day 33 and 36, only *HSP70* induced with a slight induction of *HSP90* at day 33.

4.4 Discussion

Hot air treatment clearly altered the expression patterns of *MaACO1* and *MaACS1* in the pulp of banana (Figure 4.3.1) which generally accumulated during natural ripening of banana (Liu et al., 1999, Roy Choudhury et al., 2012). These genes exhibited in hot air treated sample after 33 days of storage, where the relative level of *MaACS1* was significantly high with respect the control. This induction of *MaACS1* in the pulp could thus associate with the ripening of heat-treated banana. Despite of that *ACS* genes were encoded by multiple genes, they are the key involved in ethylene synthesis (Barry et al., 2000). On the contrary, hot air temporary induced *MaACS2* expression after storage for 1 day. This induction was presumably activated by stresses (Wang et al., 2002) as well as the banana pulp subjected to injuries (Liu et al., 1999). Our result of *MaACO1* gene with relative expression level 0-6 in the control group (Figure 4.3.1) had the similar pattern to that observed in the study of Han et al. (2016). At day 33 and 36, *MaACO1* showed relative high

expression in the pulp of hot air treated banana, which was associated with the ripening. This result suggested the *MaACO1* gene could recovered during storage. The reversible expression of *MaACO1* was previously reported by Lurie et al. (1996) that mRNA of the gene recovered after transferring tomato from heat treatment. In the previous study, Suzuki et al., (2005) found that hot air at 50°C for 2 hours delayed the expression of *ACO1* gene and ultimately suppressed *ACS1* gene throughout the storage period of broccoli. Taken together, from our results we suggested that hot air treatment inhibited the expression of *MaACO1* and *MaACS1* in the pulp of bananas, thus it restricted the ripening to delay.

In the genome of banana, there are 1, 4 and 1 homologues genes of *MaPME*, *MaPG*, and *MaPL* genes, respectively have been found. From our results, hot air treatment significantly suppressed the expression of *MaPME*, *MaPG*, *MaPL* in the pulp of banana during ripening (Figure 4.3.2). PG is responsible for pectin polymerization and solubization that required a prior action of PME to de-esterified pectin (Brummell and Harpster, 2001). From the results of our study, the transcripts of *MaPME* highly accumulated at an early storage of day 3 corresponded with an increase in the expression of *MaPG* in the pulp of banana (Figure 4.3.2). In our results, inhibition of *MaPG* expression was observed immediately after removal of the heat, whereas, *MaPME* was subsequently inhibited at day 3 of storage (Figure 4.3.2). Kagan-Zur et al., (1995) suggested that *PME* gene was less sensitive to heat stress compared to *PG* genes. It was also reported that banana *PME* gene was thermally stable in comparison with the other fruit (Le Nguyen et al., 2002). Heat treatments have been found temporary repress *PG* gene expression in strawberry

treated with hot air (43°C for 3 days) (Martínez and Civello, 2008) and peach fruit treated with hot water (60°C for 20s) (Spadoni et al., 2014). In our observations, hot air treatment significantly inhibited *MaPG* expression until day 9 of storage. High temperature was also reported to inhibit gene expression involved in ethylene production and cell wall dissembles (Lurie et al., 1996). *PG* gene expression has been reported to be ethylene-regulated by both endogenous and exogenous ethylene (Sitrit and Bennett, 1998, Tacken et al., 2010, Li et al., 2013). Thus, besides hot air treatment directly inhibited the expression of *MaPG* in banana, the disruption of ethylene synthesis genes (*MaACS1*, *MaACO*) during ripening (Figure 4.3.1) could also restrict the expression of *MaPG* in the pulp. In banana fruit, it was reported that softening occurred by concerted action of at least 4 *PG* genes (Asif and Nath, 2005). From day 15, the expression of *MaPG* was gradually increased with high expression of *MaPME* gene in hot air treated banana, which may influence to soften of banana pulp. However, Hall et al. (1993) discussed that the antisense of *PME* gene less affected on softening in transgenic tomatoes. Brummell and Harpster (2001) also suggested that the suppression of *PME* genes does not affect the firmness while suppression of *PG* could slightly reduce hardness. Thus, by the effects of hot air treatment, destruction of *MaPME* and *MaPG* may less influence pulp softening of banana. Consequently, by quantitative RT analysis of Asif et al. (2014) showed *PL* and *expansin* genes are significantly up-regulated during ripening of banana. The authors suggested that these may play important roles in the softening of the banana. We found that the relative expression of *MaPL* was high in the pulp of banana (Fig. 4.3.2). This observation concurs with Medina-Suárez et al. (1997) and

Kesari et al. (2007) that *PL* genes accumulated after post-climacteric phase and abundant in the pulp of banana at the late period of storage. In our experiment, hot air treatment strongly suppressed *MaPL* expression during the storage. It was reported in strawberry that postharvest softening was reduced approximately 10-35% in *PL*-antisense fruit (Jiménez-Bermúdez et al., 2002). Our result suggested that inhibition of *MaPL* expression significantly influenced decreasing of pulp firmness in hot air treated banana.

Recently, expansin is being addressed as an essential function for softening, is fruit-specific and highly expresses when fruit undergoes rapid softening (Rose et al., 1997, Brummell and Harpster, 2001). Surprisingly, by analyzing of *MaExp1* and *MaExp2* in the pulp of bananas, we observed that these genes mainly expressed at day 9 and 15 after storage (Figure 4.3.3). The similar pattern also found in the study of Han et al., (2016) was well as Trivedi and Nath (2004). *MaExp1* and *MaExp2* were shown to high express in the pulp of banana at the late stage of storage or post-climacteric period, and they are linked with the process of softening. With no information of the effects of heat treatment on *expansin* gene in banana, the results of our study addressed significant suppression of *expansin* genes expression in banana (Figure 4.3.3). This result was agreed with the investigation of Dotto et al. (2011) that heat treatment remarkably decreased *FaExp1*, *FaExp2* and *FaExp6* contributed to delay softening in strawberry fruit. Additionally, Martínez and Civello (2008) also reported that heat treatment temporary reduced the expression of *FaExp2* in strawberry. Multiple *expansin* genes were suggested to be required for softening of banana (Asha et al., 2007). Moreover, *expansin* has a function to

facilitate polyuronide depolymerization substantially by a pectinase late in ripening and suppression of these genes was found in firmer transgenic tomato (Brummell, 1999). Thus the effects of hot air treatment on suppressing *expansin* gene expression could subsequently limit pulp softening of bananas. Overall, hot air treatment affected the expression of cell wall-related genes differently, particularly *MaPL* and *MaExp1* and *MaExp2*. Thus the results suggested that the disruption of *MaPL, MaExp1 and MaExp2* gene expressions contributed to delaying in pulp softening of banana.

HSP70 and *HSP90* are high molecular HSPs which response to abiotic stress by accompanying the signal transduction and transcription activation of the synthesis of other members of HSPs (Wang et al., 2004). Those high molecular HSPs possibly have a critical contribution to survival and recovery from the heat stress (Waters et al., 1996). Spadoni et al. (2014) reported that hot water treatment did not clearly induce *HSP70*, *HSP90* in peach fruit. However, hot air treatment was strongly and temporary induced *HSP70* and *HSP90* in the pulp tissue of bananas fruit (Figure 4.3.4). In papaya fruit following heat treatment at 38°C for 2 hours, it was also reported that high molecular weight protein of 70-Kda was temporary induced (Paull and Chen, 1990). *HSP70* in the plant has been reported to play a protective role in thermotolerance and work on heat shock response (Lee and Schöffl, 1996). As regarded the HSPs act by altering normal pattern of genes expression (Timperio et al., 2008) and Paull and Chen (2000) demonstrated that HSPs induced by heat treatment were associated with thermotolerance. In contrast, the accumulation of *HSP90* was suggested to have role in banana disease resistance

(Wei et al., 2017). Thus, in our results, it was suggested that induction of *HSP70* by hot air treatment in the pulp of banana altered other gene expression during storage by *HSP70*. Hence, the hot air treatment at 50°C for 10 min that used for insecticidal heat treatment may potentially induce thermotolerance in banana and lead the fruit to ripen normally.

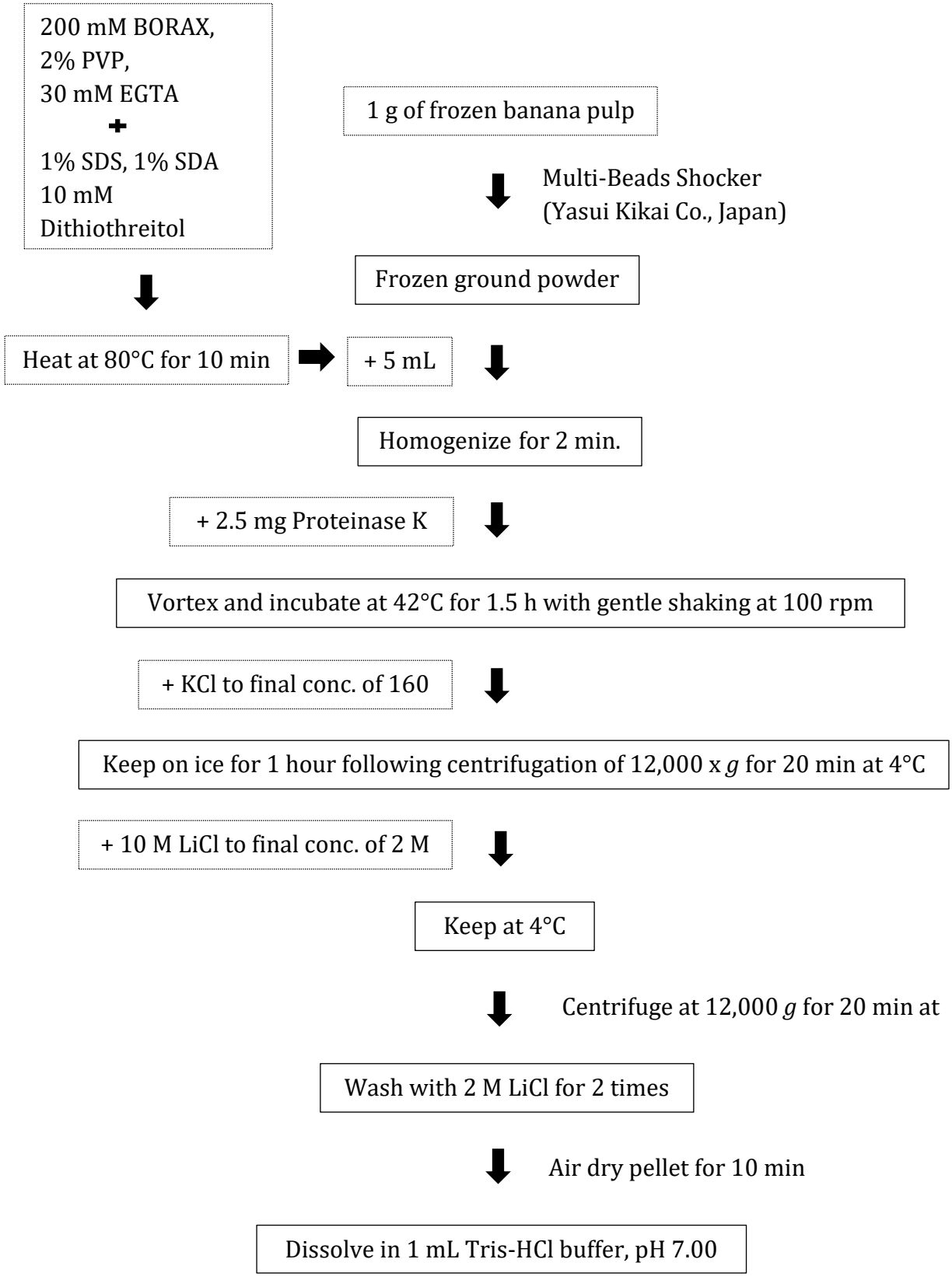
Conclusion

Hot air treatment at the temperature of 50°C for 10 min strongly inhibited the expressions of gene involved in ethylene synthesis in the pulp of banana. *MaACS2* was induced temporary just after the storage which may be stress responses to heat rather than the ripening. Thus, the inhibition of *MaACO1* and *MaACS1* indicate the ripening of banana. In addition, hot air treatment clearly exhibited the suppression of increase of cell wall modifying genes (*MaPME*, *MaPG*, *MaPL*, *Maexp1* and *Maexp2*) in the pulp of bananas. Particularly, *MaPL* and *MaExp1* and *MaExp2* genes during ripening of banana. Thus, not only the inhibition of *MaPME* and *MaPG*, but the suppressions of *MaPL*, *MaExp1* and *MaExp2* genes by hot air treatment may a major contribution to delay softening. At last, hot air treatment apparently induced the accumulation *HSP70* and *HSP90* in the pulp of banana. The accumulation of *HSP70* gene suggests that thermotolerance induced by hot air treatment whereas *HSP90* seemed to indicate heat induced stress in the pulp of banana. Hence, hot air treatment which could be used for insecticidal heat treatment of green mature banana also potentially induces thermotolerance in banana and delayed ripening and softening of banana. Further investigations are necessary for understanding of thermotolerance after hot air treatment.

Table 4.2.1

All gene sequences used in this experiment are previously designed by Amnuaysin *et al*. (2012) (*MaPG*, *MaPG*, *MaPME*, *MaAct*), Hubert and Mbéguié-A-Mbéguié (2012) (*MaACO1*, *MaACS1*, *MaACS2*), Promyou *et al*. (2008) (*HSP70*), Chen *et al*. (2009) (*HSP90*) and Wang *et al*. (2006) (*MaExP1*, *MaExP2*), which have been registered on NCBI, except *HSP90.*

.Genes		Primers (5'-3')	Accession numbers
MaPG	F	CGGATGAGCAATGTTTCCAACCCA	EU269469
	R	ACATGGAGAACTGTCGCTGCAAGA	
MaPL	F	AAGACCTGGTTCAGAGGATGCCAA	AF206319
	R	TGGCTGTTTATAGTGGGAGCAGCA	
MaPME	F	TGTCCAATGTGTCAAAGCCAGTGC	FJ264505
	R	TGGAATGCAAATCCGGAATGGTGG	
MaAct	F	TGTAGGTGATGAGGCCCAATCCAA	AF246288
	R	ATCTTCTCCCTGTTCGCTTTGGGA	
MaACO1	F	AAGCTCTACGTCGGGCATAA	AY804252
	R	GACAGCTTCCTAACGCGAAG	
MaACS1	F	AGAACTCCTCCTACTTCGAT	Y15739
	R	ATGATAGTCCTGAAAGTTGG	
MaACS2	F	TGCGGCCTTGTTCTGCTGGG	AB21907
	R	AAACCACCCCGGTTCGTCGC	
MaHsp70	F	TGAGGAGCTCAACATGGACCTG	X54030, X67711, X73472
	R	AGGTCCTGCACCTTCTCATTGC	
MaHSP90	F	CAGAGTTGCTAAGGTTCCA	-
	R	TGTTCGCAGTCCATCCATAC	
*MaExp*1	F	TGTGTGAAGAAGGGAGGCGTAA	AY083168
	R	ACTTCGGCCTCAGCGGG TGCTA	
*MaExp*2	F	AAGTGTCAGGGGAGCGGAGGCATCA	AF539540
	R	AATCCAATTGAAGGTTGGTGGACAC	


200 mM BORAX,
2% PVP,
30 mM EGTA
+
1% SDS, 1% SDA
10 mM
Dithiothreitol
1 g of frozen banana pulp
Multi-Beads Shocker
(Yasui Kikai Co., Japan)
Frozen ground powder
Heat at 80°C for 10 min
+ 5 mL
Homogenize for 2 min.
+ 2.5 mg Proteinase K
Vortex and incubate at 42°C for 1.5 h with gentle shaking at 100 rpm
+ KCl to final conc. of 160
Keep on ice for 1 hour following centrifugation of 12,000 x g for 20 min at 4°C
+ 10 M LiCl to final conc. of 2 M
Keep at 4°C
Centrifuge at 12,000 g for 20 min at
Wash with 2 M LiCl for 2 times
Air dry pellet for 10 min
Dissolve in 1 mL Tris-HCl buffer, pH 7.00

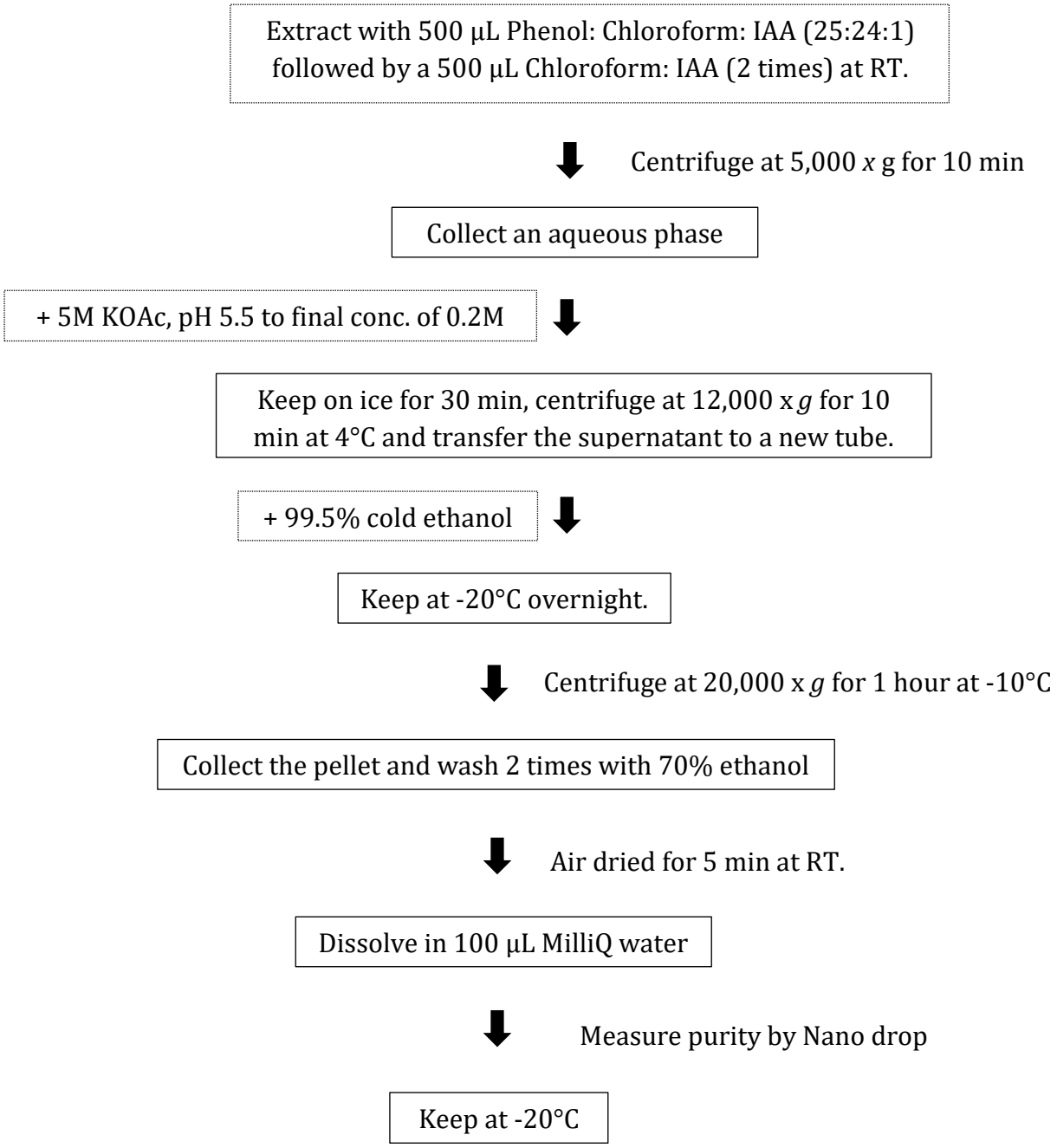


Figure 4.2.1 A flow chart of RNA extraction procedure from pulp tissue of banana.

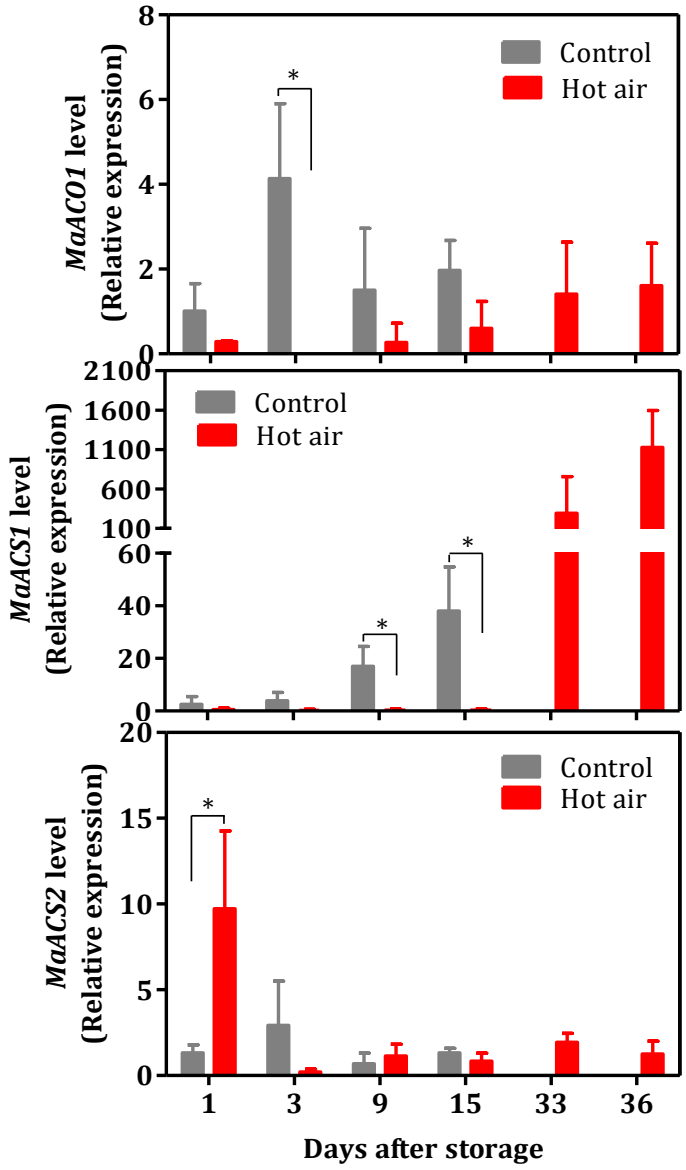


Figure 4.3.1 Expressions of ethylene biosynthesis gene (*MaACO, MaACS1, MaACS2*) in the pulp of bananas subjected to the hot air treatment (50°C, 10 min) or control treatment (room temperature, 10 min) then stored at 20°C. Data are means ± S.E. of three replications. Significant between-treatment differences ($P \leq 0.05$) are indicated by asterisks.

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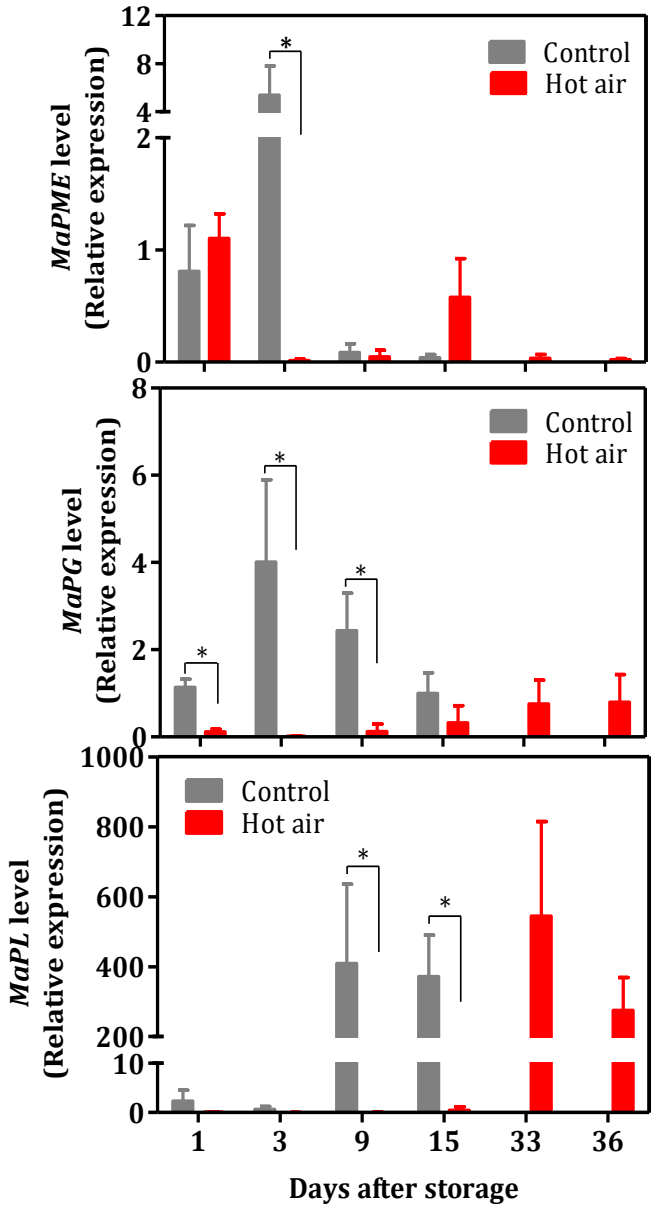

Figure 4.3.2 Expressions of Cell wall degrading genes (*MaPME, MaPG, MaPL*) in the pulp of bananas subjected to the hot air treatment (50°C, 10 min) or control treatment (room temperature, 10 min) then stored at 20°C. Data are means ± S.E. of three replications. Significant between-treatment differences ($P \leq 0.05$) are indicated by asterisks.

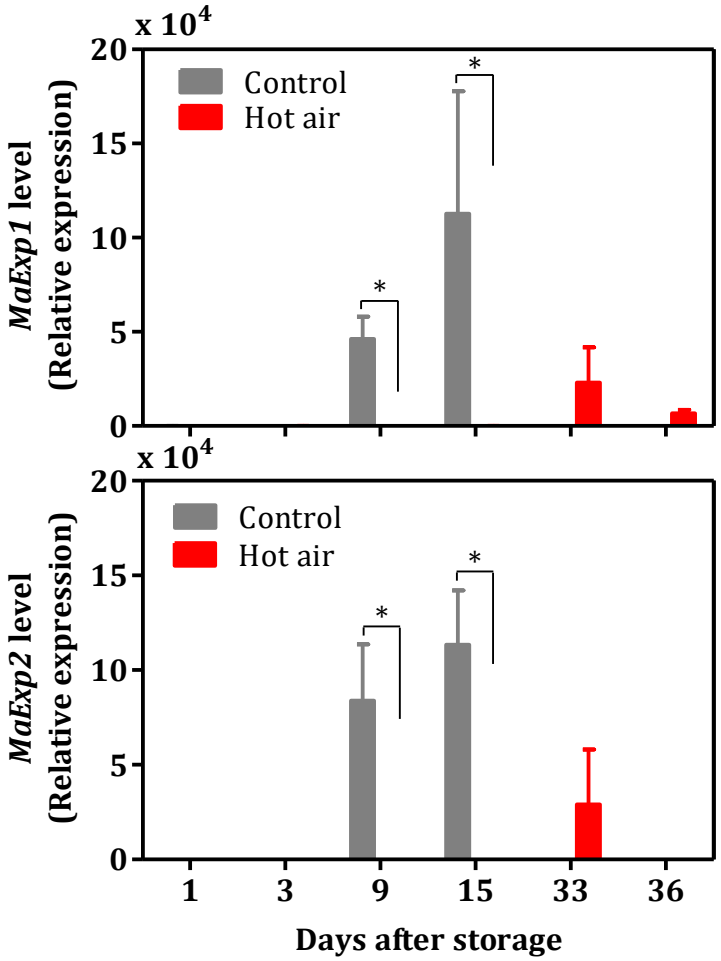

Figure 4.3.3 Expressions of expansin gene (*MaExp1, MaExp2*) in the pulp of bananas subjected to the hot air treatment (50°C, 10 min) or control treatment (room temperature, 10 min) then stored at 20°C. Data are means ± S.E. of three replications. Significant between-treatment differences ($P \leq 0.05$) are indicated by asterisks.

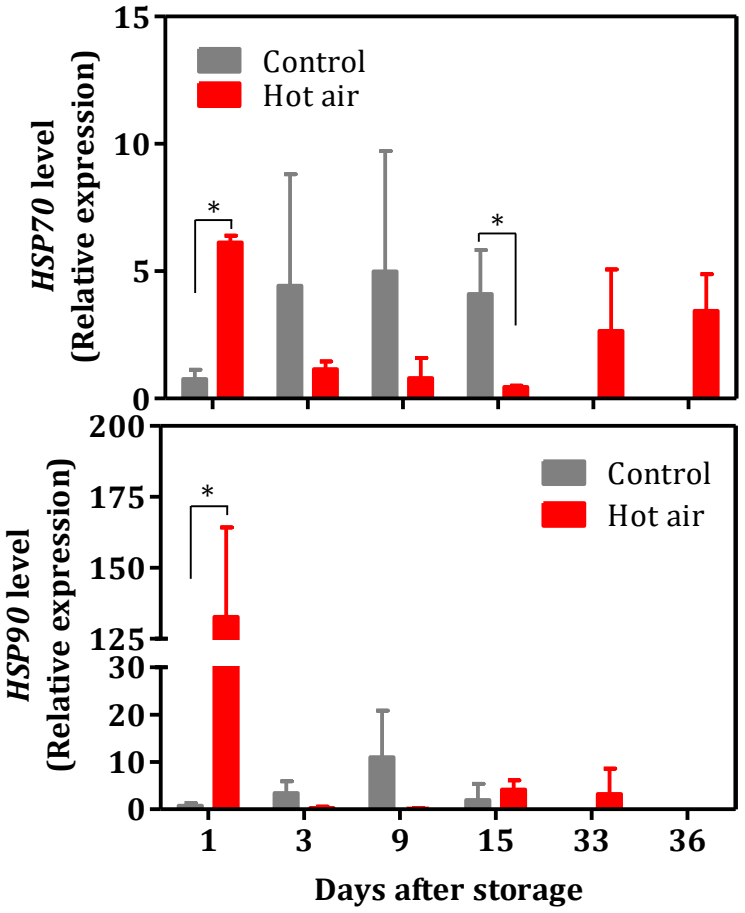

Figure 4.3.4 Expressions of heat shock proteins (*HSP70 and HSP90*) in the pulp of bananas subjected to the hot air treatment (50°C, 10 min) or control treatment (room temperature, 10 min) then stored at 20°C. Data are means ± S.E. of three replications. Significant between-treatment differences ($P \leq 0.05$) are indicated by asterisks.

CHAPTER V

General discussion

Bananas are important tropical crops widespread worldwide. Because the importation of unprocessed bananas is affected by contaminants, an inspection is requested before their shipment or during their post-entrance quarantine, in accordance with the Law of Plant Protection issued by the Ministry of Agriculture, Forestry, and Fisheries of Japan. Conventional fumigants have been used as quarantine treatments and a high volume of pesticides is required for the exportation of fresh fruit, and such high levels of chemicals and their residues might, therefore affect the environment and human health. Heat has been presented as an alternative phytosanitary treatment to the above methods, as it is an interesting chemical-free applicable technology. Hot water immersion is generally used for disinfecting bananas at, for instance, 50 ± 2°C for 5 min (Hassan et al., 2004), 45°C for 20 min (Kyu Kyu Win et al., 2007), and 45–50°C for 20 min (Reyes et al., 1998). However, only Wall (2004) applied hot water immersion in 'Brazilian' bananas to eliminate surface insect pests at 47°C, 49°C, or 51°C for 10, 15, or 20 min. The present study is the first report concerning hot air and hyper vapor heat treatment of imported bananas, most belonging to the Cavendish subgroup in Japan. These treatments at 50°C for 10 min led to 100% insect mortality in mealybugs (Nakamura et al., 2008, 2011). However, banana fruit ripening, including pulp softening, seems to be delayed by the hyper vapor heat treatment (Nakamura et al., 2012). Although hot treatment at 50°C for 10 min is a promising insecticidal treatment and beneficially inducts thermotolerance (Hara et al., 1997), its effects

are not known for Cavendish banana. Thus, it was essential to access the effects of hot air treatment on this type of banana and study its physiological changes in response to heat for further developing insecticide technology.

Our study aimed to investigate the effects of hot air treatment on the factors involved in banana fruit softening and ripening. Hence, we conducted the three experiments described in Chapters II, III, and IV. The purpose of the first topic in Chapter II was to clarify the effect of hot air treatment on the pulp softening of bananas ripened by commercial ethylene application. However, because ethylene induces ripening, this could confuse the explanation of the response to heat. Hence, in the second topic of Chapter III we conducted an experiment without using exogenous ethylene. Finally, in Chapter IV we studied banana's thermal response at the molecular level by analyzing the expression of the genes involved in ethylene synthesis and fruit softening, and heat shock proteins. Results of these experiments suggested that the hot air treatment mainly affected fruit ripening, including pulp softening and other parameters. The effects of hot air treatment are demonstrated in Figure 5.1, where responses are divided into: 1. ripening; 2. softening; and 3. other parameters.

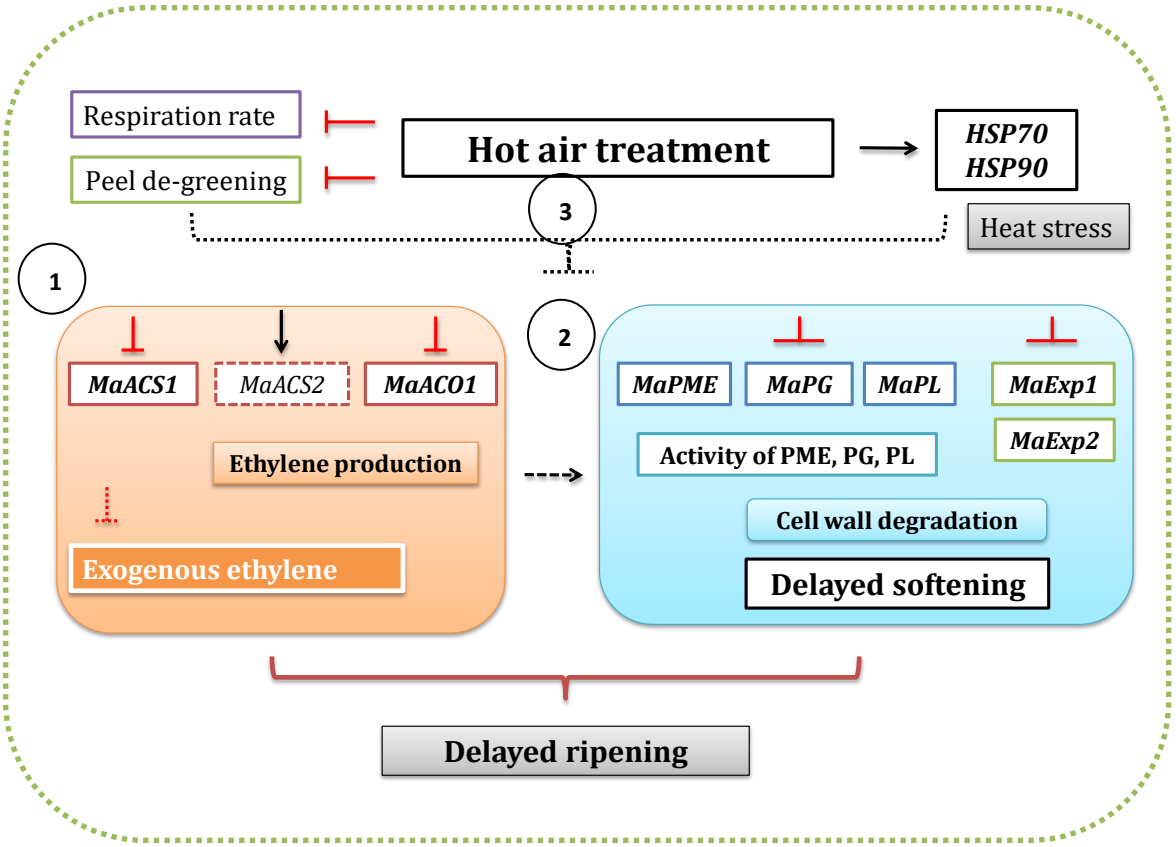

Figure 5.1 The effects of hot air treatment on banana fruit.

Ripening: banana ripening is ethylene-dependent and endogenous ethylene, as well as commercial ethylene, can initiate the ripening process. Ripening inhibition is regulated by ethylene perception.

- **Ethylene synthesis:** ethylene's biosynthetic pathway includes the enzymes ACS and ACO that regulate ACC content (Wang et al., 2002). Our study confirmed that hot air treatment substantially inhibited ethylene production and the expression of *MaACO1* and *MaACS1* in banana fruits (Figure 5.1). Results suggested that the suppression of *MaACO1* and *MaACS1* expression in the pulp corresponded to the inhibition of ethylene production thereby delaying fruit ripening. This agreed with the inhibition of ethylene production due to the suppression of *BO-ACS1*

expression reported for harvested broccoli heat-treated at 50°C for 2 h (Suzuki et al., 2005). We also observed that, in hot air treated-banana fruits, *MaACO1* and *MaACS1* recovered their expression levels after storage for 33 days, together with ethylene production. Biggs et al. (1988) showed that ethylene recovered from the marked reduction during high temperature-incubation upon removal to ambient temperature, although ethylene production may take days to be initiated in case of heat stress above 45°C (Paull and Chen, 2000). Additionally, the induction of *MaACS2* we observed at day 1 after the treatment did not indicate fruit ripening was initiated, but addressed other stresses in the fruit pulp similar to wounding response (Liu et al., 1999).

- Sensitivity to ethylene: comparing the non-ethylene treated bananas in Chapter II to the ethylene treated bananas in Chapter III, the hot air treated-group exposed to ethylene ripened for 20 days whereas the non-exposed group ripened for 33 days. However, the experiments presented in Chapters II and III are different. Although it has been suggested that hot air treatment reduces banana fruit sensitivity to ethylene, our results suggest that it might disturb ripening related receptor. Thus, exogenous ethylene might have partially induced ripening of hot air-treated banana.

The heat-inhibition of ACO and ACS has been investigated in papaya and mango (Chan, 1986, Ketsa et al., 1999). In our study, the suppression of *MaACO1* and *MaACS1* expression probably limited the activity of their encoded enzymes, ACO and ACS, respectively. Yang et al. (1990) reported the decreased production of ACC and

ethylene and a reduced sensitivity to ethylene during ripening in tomatoes incubated at 30°C. However, our study revealed that the hot air treatment reduced banana fruits sensitivity to ethylene, even after exogenous ethylene treatment. Xiao et al. (2013) reported that ethylene response factors (ERF) of banana might be involved in the transcriptional regulation of ethylene biosynthesis genes, although their function is still unclear. Ethylene receptors in banana peel might also be involved in this transcriptional regulation, but further studies are needed. Overall, the effects of hot air treatment reported in the present study indicate that disruption of ethylene production mainly occurred due to the suppression of ethylene biosynthesis genes, resulting in a delayed ripening.

Softening: softening is a complex enzymatic and non-enzymatic mechanism and its processes in the textural changes observed in banana fruit are not clearly understood. Banana is a starchy fruit and its pulp softening seems to be mostly due to the degradation of primary cell wall components and starch (Kojima et al., 1994). Prabha and Bhagyalakshmi (1998) described that enzymes related to pectin loosening might play a significant role in softening while starch degradation may contribute to the cell structure loosening. Fruit softening leads to textural changes during ripening due to the action of pectin modifying proteins involving PME, PG, and PL that affect the integrity of cell-to-cell adhesion or middle lamellas, and the presence of expansin mediates or control their action and subsequent reactions (Brummell, 1999, Brummell and Harpster, 2001, Marin-Rodriguez et al., 2002). Our results showed that hot air treatment inhibited the activity of PME, PG, and PL, in agreement with previous studies evidencing delayed fruit softening (Luo et al.,

2009, Mirshekari et al., 2015). Because PMEs de-esterifies pectic substances before the action of PGs and PLs, the inhibition of these enzymes contributes to the delay of pulp softening in banana fruits. The suppression of *MaPME*, *MaPG*, and *MaPL* expression was also observed in hot air treated-bananas. However, the expression patterns of some genes were not correlated to the activities of their encoding enzymes. These results might be due to gene expression being generally induced before enzymes increasing their activity or to enzymes being encoded by multiple genes. Thus, the activity of PME, PG, and PL might be mainly regulated by other genes. Surprisingly, the analysis of the expressions of the genes involved in cell-wall degradation in banana pulp tissue revealed that *MaPL* was abundantly expressed in ripe or soften pulps, in agreement to that found by Medina-Suárez et al. (1997). Moreover, the mRNA transcripts corresponding to the *expansin* genes *MaExp1* and *MaExp2* were abundant in ripe banana fruits (Trivedi and Nath, 2004). Although hot air treatment led to the suppression of *MaPME* and *MaPG* expressions in the pulp of banana, *expansin* genes facilitated the accessibility of cell-wall modifying enzymes (Cosgrove, 2000). Marin-Rodriguez et al. (2002) pointed out that *PL* genes play more important roles than other genes in fruit softening. Thus, hot air treatment might have inhibited *MaPG*, *MaPME, MaPL, MaExp1,* and *MaExp2* reducing the activities of cell-wall degrading enzymes and thus delaying banana pulp softening. In fact, this is the first evidence that the suppression of *MaPL*, *MaExp1*, and *MaExp2* expression in the pulp of hot air treated-banana fruits may contribute to the inhibition of pulp softening.

- Other parameters: the hot air treatment also influenced the development of the yellow color and the black spotting of banana fruits peels during ripening, as a slower decreasing rate of hue angle values were registered in hot air treated- than in control fruits. Heat treatment has been reported to inhibit banana fruit peel spotting through several enzymatic reactions (Kamdee et al., 2009). Similarly, a reduction of enzyme activity at higher temperature was suggested to be related to chlorophyll degradation (Yang et al., 2009). Funamoto et al. (2002) reported that heat treatment could reduce chlorophyll degradation due to the suppression of chlorophyll degrading enzyme activities. Thus, the delay of peel de-greening process in hot air treated-banana fruits might be related to the inhibition of chlorophyll degradation enzymes. In addition, this treatment might be applied to reduce postharvest disorders in raw commodities susceptible to black spot development and provides a tool for studying the pigment biosynthesis pathway.

It is clear that the hot air treatment temporarily accumulated HSP70 and HSP90 in the pulp of banana, possibly reflecting the plant defense responses to heat, as HSPs have been related to plants' thermotolerance (Chan and Linse, 1989). Thus, the hot air treatment has the advantage of inducing thermotolerance in banana fruits, providing relevant information regarding the response of banana to thermal conditions, which might be used for improving heat treatment and the study of physiological and biochemical changes in this fruit crop.

Overall, hot air treatment is assumed as a whole system inhibiting both physiological and molecular changes in banana fruits (Figure 5.1), as the inhibition

of ripening by the hot air treatment suppresses physiological changes as well as transcriptional changes in genes (Hoeberichts et al., 2002). Most of the genes related to banana ripening are regulated by ethylene (Kesari et al., 2007). Because *PG*, *PL,* and *expansin* genes in banana are regulated by ethylene (Sitrit and Bennett, 1998, Inaba et al., 2007), their enzyme activities were suppressed by the inhibition of ethylene biosynthesis. The hot air treatment also directly delayed pulp softening. Thus, the expressions of *MaPG*, *MaPL,* and *expansin* genes were also limited by the inhibition of ethylene biosynthesis, resulting in delayed softening. The induction of HSPs due to alterations in the pattern of gene expression (Timperio et al., 2008) led to the accumulation of HSP70 and HSP90, probably associated to a delay in the patterns of gene expression generally observed in softening and ripening processes.

Postharvest heat treatment might be a useful way to effectively eliminate insect pests in bananas as an alternative to the use of fumigants, similar to quarantine treatment at 50°C for 10 min used by Nakamura et al. (2008). Based on our results, the hot air treatment also has the advantages of maintaining pulp firmness and prolonging the shelf life of banana fruits meaning their transportation period might take longer than usual. Many fresh commodities imported by airplane are costly while fresh goods carried by ship are cheaper but take longer. Nevertheless, hot air treatment may also influence the flavor and the aroma of banana fruit. Thus, further research is recommended. Still, the effects of hot air treatment, which is a promising quarantine treatment for bananas, reported in the present thesis evidence the physiological and the biochemical changes occurring at

the molecular level, enabling to better understand the ripening and heat responses of banana fruits.

Summary

Postharvest heat treatments have been developed as chemical-free quarantine treatments requiring an optimum temperature condition to control insect pests without damaging the host. In banana fruits, heat treatments at 50°C for 10 min was efficient for mealybug control (Nakamura et al., 2008, 2011). However, the effects of this treatment on fruit quality, specifically on pulp softening, an important indicator of banana ripening, were not clearly investigated. Based on the results of the present study, the effects of hot air treatment can be assumed as a whole system because: 1. the treatment immediately inhibited respiration rate, thereby reducing metabolic activities; 2. the treatment induced stresses in banana pulp, possibly due to the immediate accumulation of HSP70 and HSP90 after the treatment, which temporarily altered ethylene biosynthesis and induced cell-wall degradation as a plant defense function by altering the regular patterns of gene expression; 3. the inhibition of the expression of *MaACS1* and *MaACO1* coincided with low ethylene production, thereby delaying ripening. Additionally, the induction of *MaACS2* indicated that hot air treatment also led to pulp stress responses. The hot air treatment also reduces banana sensitivity to exogenous ethylene, which might be linked to the disruption of ethylene receptors (Pech et al., 2012); 4. peel de-greening was delayed, which might have been due to the treatment or to ethylene biosynthesis inhibition; 5. the treatment inhibited *MaPME, MaPG, MaPL, MaExp1,* and *MaExp2* at the molecular level disturbing the activities of PME, PG, and PL. All these genes are ethylene-dependent, except *MaPME*, and the inhibitory effects of hot

air on ethylene production might have indirectly inhibited banana fruit softening or delayed this process.

Our findings provide insight into the effects of hot air treatment at 50°C for 10 min on banana pulp, which is a promising postharvest technology treatment. This treatment also proved to prolong banana fruit shelf life, which would be beneficial for preserving fruit quality during transportation.

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