BIOCHEMISTRY AND GENE EXPRESSION OF BANANA PEEL BLACKENING DURING LOW TEMPERATURE STORAGE

INTRODUCTION

Banana (*Musa* spp.) is an important commercial fruit crop in world trade. Most cultivars of the edible bananas are triploid and are derived from *acuminata* (A), and *balbisiana* (B), genomes of *Musa acuminata* and *M. balbisiana* (Simmonds and Shepherd, 1955). Cv. Hom Thong (*Musa acuminata,* AAA Group) and cv. Namwa (*Musa balbisiana*, ABB Group) bananas are commercially grown in Thailand. However, the exportation of banana is very limited due to many problems associated with postharvest quality of banana. One of the main postharvest problems is the banana fruit can not be stored for prolonged periods during shipment at temperature below 12-14^{o}C. This is caused by rapid peel blackening and other symptoms of chilling injury (CI), such as failure to ripening, hardening of the central placenta, loss of flavor, clear latex, subepidermal brown streaking and appearance of water-soaked areas (Grierson *et al*., 1967; Nguyen *et al*., 2003).

One of the primary physical events leading to chilling injury is the phase transition (from liquid to gel) in the polar lipids of the plasma membrane and other membranes. This transition is related to loss of activity of membrane-bound enzymes and loss of membrane semi-permeability. A higher proportion of unsaturated fatty acids in the polar lipids generally results in lowering of the transition temperature, and in lowering the temperature at which chilling injury occurs (Ariizumi *et al*., 2002). The changes in membrane properties are suggested to result in loss of membrane semi-permeability, thus allows leakage of compounds to other cell compartments. Blackening as a result of chilling injury seems to be the main result of an interaction between free phenolics and the activity of catechol oxidase (CO) which also called tyrosinase or polyphenol oxidase (PPO). Normally, enzymes and substrates are believed to be located in separate cellular compartments but come into contact after the membrane disruption (Robinson and Dry, 1992; Hind *et al*., 1995).

Membranes can also become disrupted as a result of lipid peroxidation. This peroxidation can occur non-enzymatically, for example by free radicals such as reactive oxygen compounds, or is mediated by enzymes such as lipoxygenase (LOX; Berger *et al*., 2001), which involves the production of reactive oxygen compounds. One of the main products of lipid peroxidation, malondialdehyde (MDA), is often measured using the thiobarbituric acid (TBA) method. As the TBA method also detects other degradation products of lipid peroxidation, the method parameter is usually called "TBA-reactive compounds" rather than "malondialdehyde". The level of TBA-reactive compounds is considered to be a useful index of lipid peroxidation under environmental stress (Queiroz *et al*., 1998). Campos *et al*. (2003) observed differences in the levels of TBA-reactive compounds in coffee leaves exhibiting a different degree of cold tolerance.

Several postharvest treatments have been designed to improve low temperature storage of chilling sensitive fruit. Hot water (HW) treatment has been shown to delay chilling injury in a number of tropical and subtropical fruits (Fallik, 2004; Lurie, 2006). The beneficial effect of heat treatment on reducing CI has been mainly reported to cause the induction of conserved polypeptide production known as heat-shock protein (HSPs) (Sebehat *et al*., 1998). The expression of the stress 70 molecular chaperones in response to heat shock is well known and it appears that low temperature exposure can also stimulate their expression to increase chilling tolerance. In banana, HW treatments were found to reduce crown rot (*Chalara paradoxa*) infection (Reyes *et al*., 1998), but the function of these proteins in protection against low temperature, if any, is unknown.

Even though a lot of studies on CI development of banana have been published, but the complex nature of this process is not well understood and a few studies have concentrated on a relation between low temperature-induced blackening and membrane-related properties (such as fatty acid composition, the activity of lipid degrading enzymes such as LOX and the level of lipid peroxidation products) including gene expression of the enzymes has apparently not been reported.

It was hypothesized that peel blackening would be correlated with each of these physiological parameters.

OBJECTIVES

1. To identify responses of banana cultivars to CI symptoms during low temperature storage

2. To determine the changes in activities of enzymatic browning (PPO, POD) and total free phenolics content including lipid composition changes and lipid peroxidation system involved in banana peel blackening during development of chilling injury

3. To study the effect of HW treatments on reducing CI of banana during low temperature storage

4. To characterize the gene expression of the genes regulating the browning and the lipid peroxidation enzymes including accumulation of heat shock protein in the banana peel after low temperature storage

LITERATURE REVIEWS

Banana

Banana is the common name used for herbaceous plants of the genus *Musa* in the family Musaceae. They are native to the tropical region of Southeast Asia. Moreover, banana is an important crop worldwide and one of most important food sources in the developing countries (May *et al*., 1995). All of the edible cultivated parthenocarpic bananas are derived from the wild diploid species namely, *Musa acuminata* (A genome, 2n = 22) or by hybridization between this species and the wild diploid species *Musa balbisiana* (B genome, 2n = 22) arising from the genomes of these two genera give rise to various types of edible bananas (Salunke, 1984). The triploid genome groups (2n = 33) such as AAA, AAB and ABB are the most important among commercially grown bananas. (Simmonds and Stover, 1987).

The banana fruit is a berry containing many ovules but lacking seed and the fruit develops by means of parthenocarpy (without fertilization). Although sterility and parthenocarpy are important factors that contribute to the desirability of banana fruits, sterility has impeded progress in breeding programs.

In the commercial production of bananas the fruit is harvested while green and ripened in market areas. The maturity at which bananas are harvested depends on the time required to get them to market. Successful marketing of edible cultivated bananas requires control over the ripening process to ensure predictable ripening and good quality ripe fruit (Kader *et al*., 2002). Bananas for export are shipped from tropical countries under refrigeration at 12 to 14°C. On arrival, they are commonly stored at 14 to 22°C under high humidity and exposed to ethylene gas at 100 to 1,000 µl/l for 24 hours to initiate ripening (Hall, 1967; Watkins, 1974).

Banana cultivars in Thailand

In Thailand, banana is one of important economic fruit crops. It is widely grown in the whole country. The fruits can be used fresh, cooked, or processed into various products. In 2002 and 2003, the amounts of export were 5,213 and 6,338 tons, respectively (Anonymous, 2003). Cv. Hom Thong (AAA) and cv. Namwa (ABB) bananas are commercially important varieties of Thailand (Abdullah *et al*., 1990).

Banana cv. Hom Thong belongs to the 'Gros Michel' group and produces medium to large fruit size. The skin is thick and turns yellow when ripe. The flesh is creamy white, fine texture, sweet and aromatic. The fingers are 15-22 cm long and 3.5-4 cm in diameter. The hands contain 12-16 fingers and the bunch has only 4-6 hands.

Banana cv. Namwa is the most widely grown banana. The fruit is small to medium in size and becomes yellow when ripe. The skin is thick and the pulp is white with a sticky consistency. The fruit is either eaten fresh or cooked (Valmayor *et al*., 1990).

Chilling injury in banana

Low temperature storage is considered to be most effective method for maintaining the quality of horticultural crops because low temperature retards respiration, ethylene production, ripening, senescence, unacceptable metabolic changes and decay (Wills *et al*., 1989). However, in long term storage at too low temperature, chilling injury symptoms can be developed, especially in tropical and subtropical fruits (Morris, 1982). According to Pantastico *et al*. (1990), CI referred to fruit disorder induced by exposure to low temperature below some critical threshold for that species or tissue (Lyons,1973).

The response of plant tissues to chilling temperatures has frequently been separated into primary and secondary responses. Two distinct possibilities are suggested as the primary event such as physical changes in membrane lipids and dissociation of enzyme or protein, and long-term response or secondary events

leading to the visible symptoms after primary sensing of chilling temperature clearly vary with tissue (Paull, 1990).

Banana fruits are susceptible to CI. Chilling damage of most banana cultivars occur at temperature below 12-13°C (Pantastico *et al*., 1990). Even a few hours of chilling temperatures can be sufficient to induce permanent or irreversible damage. CI can occur in either unripe or ripe fruit. It can cause a lowering of market quality and value or total loss. CI can easily be avoided in *Musa* cultivars/hybrids by simply limiting storage or handling to temperatures above the critical threshold. Chitrakoolsup (1982) reported four banana cultivars grown in Thailand harvested at unripe stage exhibited CI symptoms after a period of 3-7 days when stored at 10°C, whereas ripe fruits showed CI symptoms after 2-3 days. It has been suggested that the temperature range 11.5-13°C was adequate to avoid the onset of CI in banana (Hatton, 1990).

Factors contributing to development of chilling injury

Many factors affect the development of CI generally following a time and temperature relationship. CI symptoms occur more rapidly at low temperature and less at higher chilling temperature. Similarly, the longer the time of exposure to chilling temperature, the more serious the response especially when the fruit returned to high temperature. The susceptibility of banana to CI also varies more with factors contributed to the development of chilling injury depending on temperature storage, duration of exposure of fruit to that chilling temperature, whether exposure of fruits (i.e. cultivar/hybrid) to a chilling temperature is continuous or intermittent, relative humidity, composition of the storage atmosphere and post-harvest treatment, physiological age, maturity or condition of the fruit exposed, relative responsiveness (or sensitivity) of the fruit (i.e. cultivar/hybrid) to chilling, cultivars and growing condition (Lyons, 1973; Morris, 1982; Saltveit and Morris, 1990). Regardless of cultivars, ripe banana is somewhat less sensitive to chilling than mature green banana (Couey, 1982).

There is a number of commonly occurring visual symptoms which are characteristic of CI in banana including surface lesions, such as pitting, large sunken areas, hardening of the central placenta, appearance of dark water-soaked areas of the peel, brown streaking in the vascular bundles, reduced latex flow and failure of fruits to ripen normally. Fruits harvested at mature but unripe stage develop a dull, grey skin color, starch is no longer converted to sugar and fruits fail to ripen in the expected pattern following removal to ripening conditions. In severe chilling eventually turns the peel brown to black. When chilling is less severe, green fruit usually show no visible effect, but on ripening, the color of the peel varies from a dull yellow to grayish-yellow or grey (Lyons, 1973; Morris, 1982; Saltveit and Morris, 1990; Snowdon, 1990; Wang, 1993). CI affects the membranes of the tissue and the browning is caused by the oxidation of polyphenols in the latex vessels associated with the vascular bundles (John and Marchal, 1995). Moreover, CI causes cell membrane damage, resulting in electrolyte leakage can be measuring by electrolyte leakage apparently increases (Woods *et al*., 1991). Furthermore, the proportion of palmitic acid in all lipid fractions increased in both peel and pulp tissues and linolenic acid markedly decreased only in the peel tissue prior to the occurrence of irreversible injury in Cavendish banana (Wang and Gemma, 1994). Wills *et al*. (1989) showed that the membrane lipids of most tropical fruits including banana undergo a phase separation change at about 10-15°C. The membrane lipids, which are more or less fluid at higher temperature, enter a gel-like state at these temperatures and become rather immobile below that critical temperature. As a consequence of this physical change, the associated membrane proteins (especially enzymes) decrease in activity and biological function.

Recently, peel discoloration in chilled banana has been shown to be mediated through changes in the phenol metabolism (Tan *et al*., 1986) and these symptoms arise from accumulation of oxidized phenolic substances in the epidermal or sub-epidermal areas, accompanied by some retention of chlorophyll (Palmer, 1971). Chilling also affects the ripening physiology by delaying the climacteric rise and producing multiple peaks of respiration (Murata, 1969) and by reducing the production of volatiles (Mattei and Pailliard, 1973).

Phenolic compounds oxidation in plant: a browning process

Phenolic compounds are widely distributed in plants and act as substrates for a number of oxidoreductase, namely, PPO and POD, which catalyse the oxidation of phenols to their corresponding semiquinones and quinones (Vaughn and Duke, 1984). Semiquinones and quinones are highly reactive species that undergo further non-enzymatic reactions. They can spontaneously react with phenols, amino acids or proteins, yielding a complex mixture of brown products (Walker and Ferrar, 1998) (Figure 1). Non-enzymatic oxidation of phenolics, such as autoxidation and chemical oxidation, can also lead to the formation of quinoidal compounds (Mochizuki *et al*., 2002). Through coupled oxidation reactions, quinines can oxidize other polyphenols that can not be directly oxidize by the enzymes, thereby forming secondary quinines, which in contribute to the formation of heterogeneous polymers responsible for the browning reaction. The composition of these brown polymeric species is extremely difficult to characterize *in vivo*. The enzymatic oxidation of polyphenols, also occurs during food processing of plant materials as well as during storage, when cell integrity is affected (Cheynier, 2005).

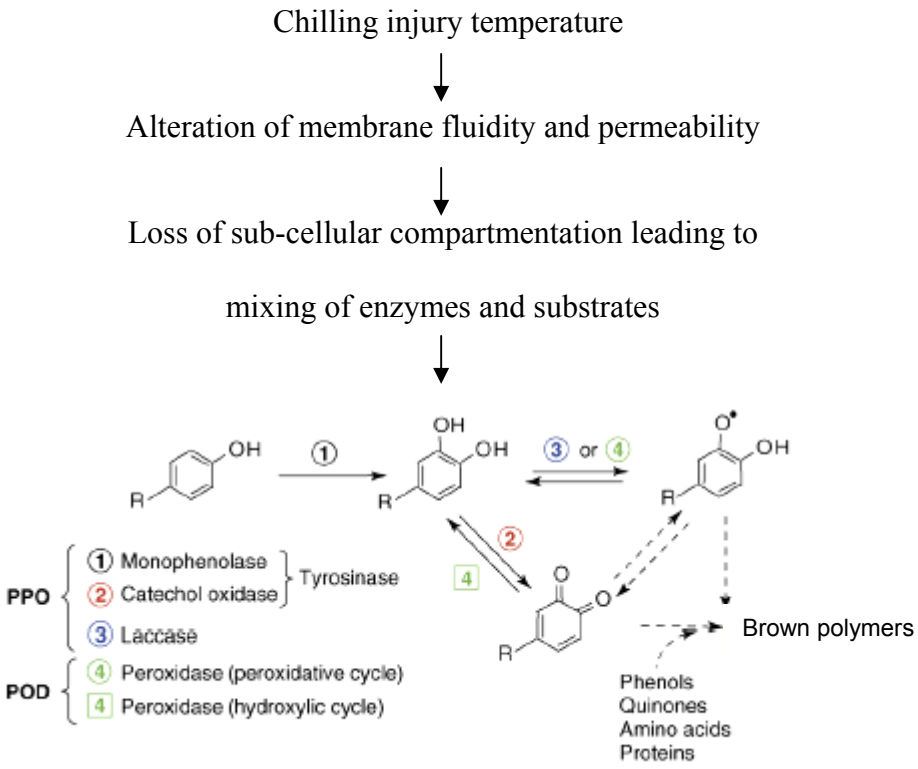

Figure 1 Mechanisms of phenolics oxidation leading to brown polymers.

Source: Modified the schemes by Pourcel *et al*. (2006)

Banana fruit tissues and particularly the peel are rich in phenolics such as 3,4-dihydroxyphenylethylamine (dopamine), 3,4-dihydroxyphenylalanine (L-dopa), tyramin (Wallrauch and Pflaum, 2005) and norepinephrine (Riggin *et al*., 1976). Dopamine is the main substrate for browning in banana. Its concentration in the pulp of unripe fruit is about 50 mg (g^{-1} pulp fresh weight), but is halved as the fruit ripens. The concentration of phenolics in the peel of green fruit is about 700 mg (g^{-1} peel fresh weight), and ripening reduces its concentration less than one-third. At the subcellular level, free phenolics are present mainly in the vacuole, but are synthesized in the cytoplasm (Vamos-vigyazo, 1981; Walker and Ferrar, 1998). Moreover, the discoloration occurs with CI development in fruits and vegetables are often associated with metabolism of phenolic compounds (Saltveit and Morris, 1990). These compounds, when oxidized by the enzyme PPO are responsible for the rapid browning of banana tissues (Palmer, 1971). Cold storage of fruits has given very

interesting data on relations with phenolic metabolism. Macheix *et al.* (1990) reported that temperature appears to be an important factor in the expression of the phenolic metabolism of a fruit either through direct, more or less specific action on certain enzymes of this metabolism, or indirectly through the overall metabolism and fruit growth and maturation. In the case of Anjou pear, during storage at 1°C, total phenol levels increase in fruit skin and flesh (Blankenship and Richardson, 1985). Similarly, increased concentration of phenolic compounds was associated with color changes in jicama (Aquino-Bolanos *et al*., 2000). Cano *et al*. (1997) also found banana freezing/thawing processes produced a significant increase in phenolic compounds in all samples. In contrast, Nguyen *et al*. (2003) found CI development in the peel of banana was highly inversely correlated with the level of phenolic compounds.

Enzymatic browning and chilling injury

Browning in some fruits and vegetables is initiated by the enzymatic oxidation of phenolic compounds. When cells are damaged by low temperature, the contents of cytoplasm and vacuoles are mixed, and phenolic compounds can readily become oxidized by air. The main enzymes covered are PPO and POD can also cause the oxidation of phenolic compounds (Mayer and Harel, 1991). The initial products of oxidation are quinones, which rapidly condense to produce relatively insoluble brown polymers.

PPO (EC 1.10.3.1) is comprised of a group of copper protein complex enzymes that catalyze the oxidation of phenolic compounds to produce brown pigments at damaged surfaces of fruits and vegetables. Generally, PPO catalyzing the oxidation of o-diphenols to the corresponding *o*-quinones and also catalyze the oxidation of monophenols to *o*-diphenols (Figure 2). In plant, PPO is encoded by nuclear genes and predominantly located in the chloroplast thylakoid membranes and in vesicles or other bodies in non-green plastids (Hind *et al*., 1995) while its substrates are located in the vacuole. It is thought that PPO mediated browning reactions occur after a loss of compartmentation. PPO activity has been shown to be highest in young tissues and meristematic regions, and gene expression generally decreases during

development and maturation of plant tissues (Boss *et al*., 1995). PPO activity is often high in developing fruits, decreasing towards maturity (Vamos-Vigyazo, 1981).

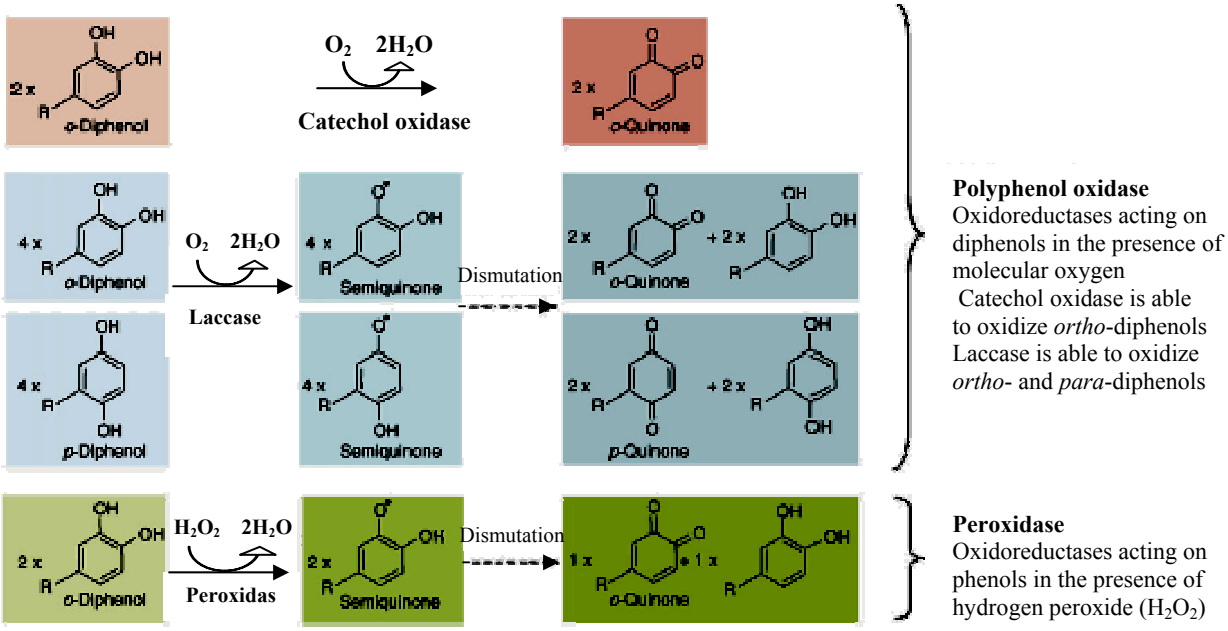

Figure 2 Enzymatic reactions for polyphenol oxidase and peroxidase.

Source: Pourcel *et al*. (2006)

According to Yang *et al*. (2000) the main PPO in banana peel is catechol oxidase (EC 1.10.3.1). The enzyme showed two bands after staining with Coomassie brilliant blue on a PAGE gel. Gooding *et al*. (2001) reported that banana flesh was high in PPO activity throughout growth and ripening. Peel showed high levels of activity early in development but activity declined until ripening started and then remained constant. In the peel and pulp of banana (*Musa sapientum* L.), dopamine was detected in large quantity (Riggin *et al*., 1976; Tono *et al*., 1999; Yang *et al*., 2000; Romphophak *et al*., 2004), and it was strongly oxidized by PPO (Galeazzi *et al*., 1981). In chilling-sensitive commodities including banana, phenolic compounds (PPO and POD substrates) also increase, promoting the development of browning during storage at low temperature (Saltveit and Morris, 1990). The report of Nguyen *et al*.

(2003) also found that increase of PPO activities may be causally related to CI induced peel browning of banana (*Musa* AA Group).

Genetic engineering also offers alternatives for producing fruits and vegetables with increased resistance to enzymatic browning. For instance, potatoes expressing sense or anntisense RNA from tomato PPO show less enzymatic browning (Coetzer *et al*., 2001). PPO gene expression is usually highest in developing tissues and meristematic regions and decreases during tissue maturation (Boss *et al*., 1995). Gene encoding PPO have been isolated and characterised from a wide range of plants and generally constitute multigene families such as PPO and POD have been maintained during evolution, suggesting that each enzyme is needed to carry out different specific functions in plants. (Cary *et al*., 1992; Newman *et al*., 1993; Thygesen *et al*., 1995). Alignments of deduced polypeptide sequences reveal the presence of two highly conserved copper-binding regions, containing conserved histidine residues, which are responsible for the binding of prosthetic copper groups essential for PPO activity. Plant PPO genes generally encode a peptide of approximately 67 kDa which gives rise to a mature protein of approximately 60 kDa following the *N*-terminal cleavage of a transit peptide during transport into the plastids (Cary *et al*., 1992; Robinson and Dry, 1992; Hunt *et al*., 1993). In tomato, the seven member of the PPO gene family are differentially induced or downregulated in various tissues and by various types of environmental stresses (Thipyapong *et al*., 2004). In banana fruit was amplified four different PPO cDNA clones (*BPO1, BPO11, BPO34 and BPO35*) and the genomic clone was found to contain an 85 bp intron (Gooding *et al*., 2001). Moreover, PPO genes were strongly up-regulated in response to chilling and wounding in pineapple (Stewart *et al*., 2001).

POD (EC 1.11.1.7) is iron-porphyrin organic catalysts that are widely distributed in plants and seem to be normal components of most plant cells (Cano *et al*., 1998) and classified according to their subcellular localization, class-I POD being intracellular, and class-III POD being secreted to the apoplast after glycosylation (Takahama, 2004). The ability of POD to contribute to enzymatic browning is related to its affinity to accept a wide range of hydrogen donors, such as polyphenols. Two possible mechanisms are proposed for POD catalyzed browning

reactions. One involves the generation of H_2O_2 during the oxidation of some phenolic compounds that is used as in a normal peroxidatic action to further oxidize the phenol, while the second involves the use of quinonic forms as substrate by POD. Both mechanisms indicate that the presence of PPO would enhance POD-mediated browning reactions (Richard-Forget and Gauillard, 1997).

Cano *et al*. (1997) reported that banana cultivars exhibit a higher POD activity could therefore be more susceptible to chilling injury problems during handling and transport. Perez-Tello *et al*. (2001) have shown that chilling injury symptom development in carambola fruit associateded with increased trend in POD activities, while iceberg lettuce treated at 4°C showed a decrease in the POD activities (Martin-Diana *et al*., 2004). Similarly Rivero *et al.* (2001) had reported a decrease in POD activity of watermelon during storage at low temperature.

Lipid composition and chilling injury

In plant tissue, most of the lipids are membrane components. The fatty acids present varying in the number of *cis*-double bonds (Ohlrogge and Browse, 1995). Chilling impairments mainly consist of alteration of metabolic processes, decrease in enzymatic activities, reduction of photosynthetic capacity and change in membrane fluidity among others (Dubey, 1997). Such changes are frequently related to an increase in membrane permeability. Previous studies reported that low temperature has differential effects on the lipid composition of membranes in chilling-sensitive and chilling-tolerant plants. For example, the proportion of unsaturated fatty acids tends to increase in the thylakoid membrane, mitochondrial membrane, and plasma membrane during low temperature stress (Xu *et al*., 1997; Santis *et al.*, 1999).
In addition, changes in membrane lipid during cold storage have been associated with increased leakage varied with the fruit, not only an increase in sterol content, palmitic acid (16:0) and the saturation index, but also a decrease in linoleic acid (18:2) content and the double bond index. Lyons *et al.* (1964) reported that the degree of unsaturation of fatty acids in membrane lipid was higher in chilling-resistant tissues than in chilling-sensitive tissues. Wang and Gemma (1994) also found the ratio of

unsaturated/saturated fatty acid in the peel tissues of banana decreased during the chilling peroid. Wang and Baker (1979) observed that the higher degree of unsaturation of fatty acids coincided with the higher chilling resistance in cucumber and sweet pepper. Polyunsaturated fatty acids, very abundant in galactolipids molecules, are the preferential substrate of peroxidation and hydrolytic enzymes (Sahsah *et al*., 1998). Malondialdehyde (MDA) is one of the final products of stress induced lipid peroxidation of polyunsaturated fatty acids (Leshem, 1987), and has been considered a marker for cold sensitivity (Queiroz *et al*., 1998). Moreover, changes in membrane lipid components associated with alteration to biophysical and biochemical membrane properties may result in cellular decompartmentation (Marangoni *et al*., 1996).

Membrane permeability and leakage

There are many symptoms of CI of horticultural crops associated with the changes in membrane permeability. Membrane permeability is an expression of the freedom with water and solutes can pass through the membrane. Increased permeability of membranes may cause the promotion of an enzyme-substrate interaction, resulting in the occurrence of browning. Electrolyte leakage is generally considered as an indirect measure of plant cell membrane damage and has been used to determine the extent of CI (Woods *et al*., 1991). King and Ludford (1983) reported that mature green tomatoes analyzed immediately after chilling showed higher electrolyte leakage in chilling-sensitive lines than in chilling-tolerant lines. Many scientists presented data indicating that increased membrane permeability and increased rates of ion leakage are associated with CI of sensitive tissue (Murata and Tatsumi, 1979; Paull, 1981; Kuo and Parkin, 1989; Saltveit, 2000a). Membrane permeability as a result of enzymatic lipid peroxidation in plants is mainly catalyzed by lipoxygenases (LOXs) (Berger *et al*., 2001).

LOX (lineolate:oxygen oxidoreductase, EC 1.13.11.12) is non-haem iron-containing dioxygenases widely distributed in the plant kingdom and with diverse

functions. These include associated with membrane deterioration in plant tissues through peroxidation of polyunsaturated fatty acids, resulting in loss of compartmentation and cell breakdown (Rogiers *et al*., 1998). In plants, linolenic and linoleic acids are the most common substrates for LOX. Oxygen can be added to either end of the pentadiene structure. In the case of linoleic or linolenic acid leads to two possible products, the 9- and 13-hydroperoxy fatty acids (Siedow, 1991). Previous work on LOX activity in guava fruit, suggests that LOX activity increases with low temperature induced fruit blackening (Gonzalez-Aguilar *et al*., 2004) same as the resulted of Xu *et al*. (2003) showed that LOX activity increased in kiwifruit after storage at 0°C for 2 days.

Several LOX cDNAs have been isolated from various sources (*Arabidopsis*, rice, wheat, barley, potato, tomato, and tobacco) that carry a putative chloroplast transit peptide sequence. Based on this N-terminal extension and because these enzymes show only a moderate overall sequence similarity of ~40% among each other. In *Arabidopsis thaliana*, at least six LOX genes have been characterized. Expression of *AtLOX1* is stimulated by the stress-related hormones abscisic acid (Melan *et al*., 1993) and methyl jasmonate (Creelman and Mullet, 1997). LOX gene expression is also regulated by different effectors such as the source/sink status (Fischer *et al*., 1999), and different forms of stress, such as wounding (Porta *et al*., 1999), pathogen attack (Melan *et al*., 1993) and low temperature (Zhang *et al*., 2006). In addition, LOX gene isolated from different plant species show differential organ specific expression (Kolomiets *et al*., 2001). In a different *Actinidia* species (A. chinensis) it has been found that *AdLox1* and *AdLox6* were substantial elevated in fruit tissues damaged by low temperature after long-term storage (Zhang *et al*., 2006).

Reduction of chilling injury by hot water treatments

The degree of CI can be reduced by several techniques. Heat treatment is one of the most simple and efficient methods. Hot water treatments have been shown to reduce CI during storage in a wide range of horticultural crops including avocado (Woolf *et al*., 1995), tomato (McDonald *et al.*, 2000), orange and citrus fruit (Porat, 2004),

grapefruit (Dafna, *et al*., 2004), cucumber (Saltveit *et al*., 2004) and pomegranate (Mirdehghan *et al*., 2007). This resistance to low temperature injury caused by heat treatments is the induction of heat shock proteins (Hsp). Collins *et al*. (1995) confirmed the direct relationship between chilling tolerance and Hsp. Class II smHsp gene expression in tomato fruit was increased by high temperature and was also expressed when the heated fruit were transferred to low temperature.

As previously described, Hsp70 might play a specific role in the acquisition of tolerance to chilling stress following heat pretreatment and having essential functions in preventing aggregation and in assisting refolding of non-native proteins under stress conditions (Kadyrzhanova *et al.*, 1998). Other reports of the effectiveness of a heat exposure in reducing chilling sensitive are the use of heat pretreatment at 38°C for 10 h induced an increase in Hsp70 expression, which paralleled the decrease in electrolyte leakage under chilling injury conditions (Zhang *et al*., 2005). An examination of the lipid composition of apple (Lurie *et al*., 1995) and pomegranate (Mirdehghan *et al*., 2007) showed that after heated fruit and stored at chilling temperature, there were more unsaturated fatty acid and maintain the ratio of higher unsaturated/saturated fatty acid in heated than in non-heated fruits. Moreover, heat treatments can affect the stability of phenolic constituents and inactivate enzymatic browning such as PPO and POD. In iceberg lettuce, heat treatments from 45 to 55°C for 1 min significantly decreased PPO activity along with the synthesis of phenolic compounds (Loaiza-Velarde *et al*., 1997). Weemaes *et al*. (1998) reported high temperature at 60-65°C inactivation of PPO in plum, apple, pear and avocado. High temperature inactivation of POD is described by biphasic linear first-order kinetics consisting of an initial rapid inactivation (Yemenicioglu *et al*., 1998; Forsyth *et al*., 1999), though it might contribute to a reduction in chilling damage in heat-conditioned fruit (Martinez-Tellez and Lafuente, 1997). In addition, regeneration of POD activity after heat treatment, due to recombination of the haem group with apo-peroxidase is a common feature reported for horseradish (Lopez and Burgos, 1995), asparagus (Rodrigo *et al*., 1997) and soybean sprouts (Lee *et al*., 1999). Therefore, long heat treatment times to ensure POD inactivation, as well as control of POD activity during further storage, are strongly recommended.

Analysis of gene expression

In all organisms, there are two major steps separating a protein-coding gene from its protein: first, transcription of the information encodes in DNA into a molecule of RNA and translation of the information encode in the nucleotides of mRNA into a defined sequence of amino acid in a protein. The process of producing a biologically functional molecule of either RNA or protein is called gene expression.

Reverse transcription polymerase chain reaction (RT-PCR)

In order to detect gene expression, the reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for the detection of low-abundance mRNA, often obtained from limited tissue samples and common use for the quantification of transcription (Weis *et al*., 1992). Quantification of mRNA can be determined by semiquantitative or quantitative methods (Ferre, 1992). Semiquantitative RT-PCR analysis can be used to compare the levels of mRNAs in different sample populations to characterize patterns of mRNA expression based on the use of an internal control, which is a housekeeping gene and/or ribosomal RNAs (rRNA). RNA, are useful internal controls, as the various rRNA transcripts are generated by a distinct polymerase (Paule and White, 2000) and their levels are less likely to vary under conditions that affect the expression of mRNAs (Barbu and Dautry, 1989). Indeed, they have been shown to be more reliable than either of the housekeeping genes. First step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA by reverse transcriptase enzyme and followed by its exponential amplification in a PCR reaction. There are three major steps in the cycling of PCR reactions: step 1 PCR denaturation at about 94°C, in this PCR step the double strand melts to single stranded DNA. Step 2 PCR annealing at about 40-65°C depending on the length and base sequence of the primers, in this PCR step the primers bind to the single strand with high specificity created in the previous PCR step. Step 3 PCR extensions at about 72°C, the PCR polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, to make two double stranded molecules (Figure 3a). The PCR steps are repeated (Figure 3b).

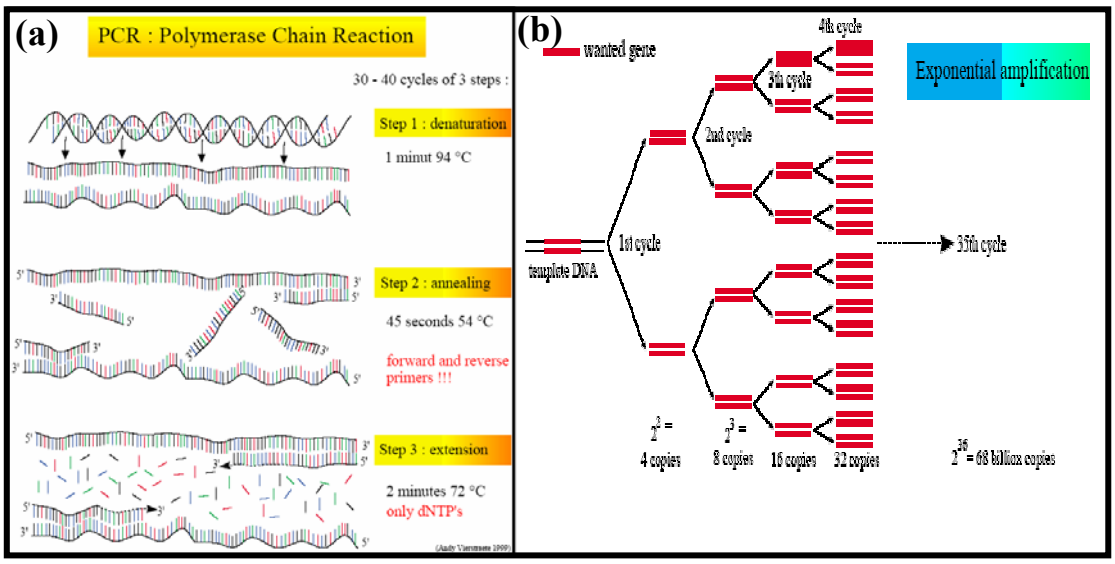

Figure 3 The different steps and the exponential amplification of the gene in PCR.

Source: Vierstraete (1999)

In addition, it is important to select the appropriate number of cycles so that the amplification product is clearly visible on an agarose gel and can be quantified, but also amplification is in the exponential range and has not reached a plateau yet. The optimal number of cycles has to be in the same range for the specific RNA of interest and the control (Marone *et al*., 2001).

Real- time PCR

Real- time PCR describes the change in expression of the target gene relative to some reference group such as untreated control or a sample at time zero in a time course study. Real-time polymerase chain reaction evolved from the polymerase chain reaction technique which was developed to allow the amplification of small numbers of DNA molecules. Real-time PCR involves first isolating mRNA from a particular cell sample before producing a DNA copy of complementary DNA of each mRNA molecule using the enzyme reverse transcriptase. The basic idea behind real-time polymerase chain reaction is that the more abundant a particular cDNA (and thus mRNA) is in a sample, the earlier it will be detected during repeated cycles of amplification. Various systems exist which allow the amplification of DNA to be followed and involve the use of a fluorescent dye such as SYBR Green which is

incorporated into new synthesized DNA molecules during real-time amplification. Real-time polymerase chain reaction machines, which control the thermocycling process, can then detect the abundance of fluorescent DNA and thus the amplification progress of a sample. Typically, amplification of a cDNA over time follows a curve, with an initial flat-phase, followed by an exponential phase. Relative concentration of DNA present during the exponential phase of the reaction is determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold detection of fluorescence background is determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold (Ct). Since the quantity of DNA doubles every cycle during the exponential phase, relative a mounts of DNA can be calculated. This data can be analyzed by computer software to calculate relative gene expression between several samples or mRNA copy number based on a standard curve (Livak *et al*., 2001).

MATERIALS AND METHODS

Experiment 1: Study on physiological and biochemical changes of two banana cultivars in relation to chilling injury

Fruit at 80% maturity (commercial maturity) of cv. Hom Thong (*Musa acuminata,* AAA Group) and cv. Namwa (*Musa* x *paradisiacal*, ABB Group) were harvested from a plantation in the Petchaburi province (Western Thailand). Dehanded bananas were placed in corrugated cardboard boxes and transported by a refrigerated truck (25°C) to the Postharvest Research Unit, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom within 3 h of harvest. In the laboratory, the hands were selected for uniformity of finger size and washed with water containing 100 mg/l sodium hypochlorite, then treated with 500 mg/l thiabendazole solution to control fruit rot and allowed to air dry at ambient temperature (29-30°C, 85% RH). Bananas were randomly placed in corrugated cardboard boxes and stored at 4 and 12°C, 85%RH. Bananas were inspected for peel blackening and peel was collected for chemical analysis, using three replications per treatment. Each replication consisted of the whole peel from 10-15 randomly sampled fruits which were pooled together, frozen in liquid nitrogen immediately after removal from the fruit for prevent oxidative damage and then stored at -80° C for later analysis. The peel material was homogenized to ensure that the 5 gram samples taken for analysis were representative. The following parameters were recorded:

1.1 CI evaluation

CI was evaluated every two days, by determining the brown area on the peel of 30 individual fruit in each treatment using a scale from 1 to 5; 1 = no chilling injury; 2 = mild injury (1-20% of fruit affected); 3 = moderate injury (21-50% of fruit affected); 4 = severe injury (51-80% of fruit affected); 5 = very severe injury (81-100% of fruit affected) as showed in Figure 4. CI severity index was calculated as follow:

$$\text{CI severity index} = \frac{\sum (\text{CI scale} \times \text{Number of fruit at the level})}{\text{Total number of fruit in each group}}$$

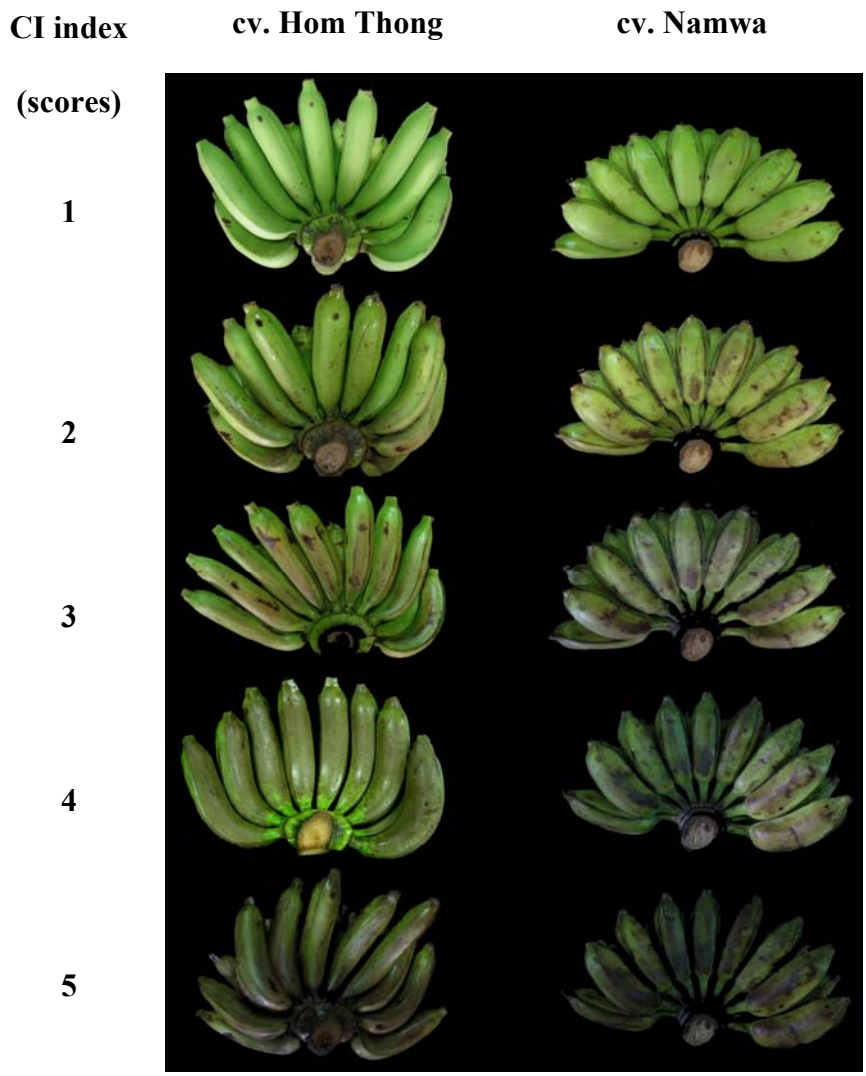

Figure 4 CI index in banana cvs. Hom Thong and Namwa.

1.2 Fruit peel color

Change in color of banana peel was determined using a colorimeter (Minolta CR300). Banana peel was measured at the middle part at both sides. L value was recorded where: L = lightness, range from 0 (black) to 100 (white).

1.3 Membrane permeability

Membrane permeability was determined based on electrolyte leakage (%). Ten discs (2 mm thickness and 15 mm diameter) were excised from peel by a cork borer, rinsed with 50 ml of distilled water. They were incubated in 30 ml of 0.4 M mannitol solution and shaken at 100 cycles per min. Initial electrolyte leakage was determined following incubate at ambient temperature for 3 h constant shaking. Electrical conductivity of the bathing solution was measured using a conductivity meter. Final electrolyte leakage was determined after autoclaved at 121°C for 1 h to release electrolytes. The percentage of electrolyte leakage was calculated by using equation: Electrolyte leakage (%) = (Initial electrolyte leakage / Final electrolyte leakage) x 100

1.4 TBA-reactive compounds

Five grams of banana peel were homogenized with 25 ml of 5% (w/v) trichloroacetic acid (TCA). The mixture was centrifuged for 10 min at 4,000 g. Thiobarbituric acid (TBA) reactivity was determined by adding 2.5 ml of 0.5% TBA in 15% TCA to 1.5 ml of the supernatant. The reaction solution was held for 20 min in a boiling water bath, then cooled quickly and finally centrifuged at 4,000 g for 10 min to clarify the solution. Absorbance was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm, calculated with an extinction coefficient of 1.55 $nmol.L^{-1}.m^{-1}$ on a fresh weight basis by the method of Guidi *et al*. (2000). The TBA-reactive compounds were calculated as follows:
TBA-reactive compounds (nmol/g FW) = (Absorbance at 532 nm - Absorbance at 600)nm/100

1.5 Fatty acid determination

Methyl esters of fatty acids were separated and quantified by the AOAC (1995) method, using a gas chromatograph (Varian, model CP 3800, Palo Alto, CA, USA) with a 60 m x 0.25 mm DB-23 capillary column and a film thickness of 0.25 mm (Agilent Technologies, Wilmington, DE, USA), coupled to a flame ionization detector. The injector and detector were maintained at 230 and 250°C, respectively. Samples were injected on the column at a split rate of 1: 50 with a helium carrier gas flow rate of 1 ml/min. All solutions were injected using three replications (each from another biological sample) and standards were also injected three times. Total saturated fatty acid is the sum of palmitic (16:0), stearic (18:0) and beheric (22:0) and total unsaturated fatty acid is the sum of elaidic (18:1), linoleic (18:2) and linolenic (18:3).

1.6 Total free phenolics

The method to measure total free phenolics was modified from Ke and Saltveit (1989). Five grams of peel tissue were homogenized with 20 ml of HPLC grade methanol; the homogenates were filtered through cheese-cloth and then centrifuged at 15,000g at 20°C for 15 min. The supernatant was taken and diluted depending on the phenolic concentration. Five ml of Folin-Ciocalteu was added to 1 ml of the diluted free phenolics extract and mixed well. Four ml of 20% of Na_2CO_3 was added and then mixed again. The mixture solution was incubated at 40°C for 30 min, and was determined photometrically. The absorbance at 280 nm was recorded, using pure dopamine as a standard.

1.7 Enzyme extraction and assay

LOX was extracted and assayed by the method described by Lara *et al*. (2003). Five grams of banana peel were homogenized in 1 ml of extraction solution containing 0.1 M phosphate, pH 7.5, 2 mM DTT, 1 mM EDTA, 0.1% (v/v) Triton X-100 and 1% (w/v) PVPP. The homogenate was centrifuged at 18,000 g for 20 min at 4°C, and the supernatant held at 0°C. LOX activity assayed by mixing 100 µl of the supernatant

with 2.5 ml of 0.1 M phosphate, pH 8, 400 µl substrate solution (8.6 mM linoleic acid, 0.25% (v/v) Tween-20, 10 mM NaOH, in 0.1 M phosphate, pH 8). Activity was measured by following the increase in absorbance at 234 nm. One unit of enzyme activity was defined as the increase in absorbance per min and per ml enzyme solution.

PPO was extracted and assayed according to a modification of the method by Yang *et al*. (2001). Five grams of banana peel was homogenized in 15 ml of 0.1 M sodium phosphate-0.1 M potassium phosphate buffer (0.1 M PB, pH 7) with addition of 0.3 g polyvinyl polypyrolidone (PVPP) for 3 min. After filtration of the homogenate through cheesecloth, the filtrate was centrifuged at 18,000 g at 4°C for 20 min. The supernatant was used for enzyme assay and protein content. The assay medium contained 1.0 ml of 0.02 M aqueous solution of dopamine, 3.9 ml of 0.1 PB (pH 7) and 0.1 ml of the enzyme solution. After incubation at 30°C for 5 min, the absorbance of the mixture at 420 nm was measured. One unit of enzyme activity was defined as an increase in absorbance per min and per ml enzyme solution (1 cm light path).

POD was extracted and assayed according a modification of the method of Kang *et al*., (2003). Crude extracts of the enzyme are obtained from 5 g of homogenized peel tissue with 0.3 g of PVPP and 15 ml of a phosphate buffer (50 mM, pH 7). The homogenized mixture was centrifuged at 18,000 g at 4°C for 20 min and the supernatant liquid was used as enzyme extract. For the activity assay, 0.3 ml of 10 mM H_2O_2 was added to1.6 ml of 10 mM phosphate buffer (KH_2PO_4) pH 7 containing 0.6 ml of 20 mM guaiacol added just before assay. This mixture was used as the substrate solution. A sample of supernatant (0.5 ml) and 2.5 ml substrate solution were mixed and the reaction was measured within 4 min, using spectrophotometer with absorbance at 470 nm as a result of guaiacol oxidation.

1.8 Protein content

In order to calculate specific enzyme activity, the protein content was measured using the method described by Bradford (1976). Four ml of Coomassie Brilliant Blue G-250 was added to 1 ml of enzyme solution. The samples were measured

at 595 nm and protein concentration was determined using bovine serum albumin standard curve.

1.9 Statistical analysis

Experimental data are the average of three replications ± standard error (SE). Analysis of Variance (ANOVA) was calculated and means separations were determined using Duncan's multiple range tests (DMRT) at $P \leq 0.05$.

Experiment 2: Study on effect of hot water treatments (HW) on reducing CI of banana during low temperature storage

In both cultivars, the hands were dipped in hot water at 42°C for the following periods 5, 10 and 15 min (water bath Model GFL 1092, Germany). Hands of banana immersed in distilled water at 25°C served as controls and randomly placed in corrugated cardboard boxes then stored at 4°C, 85%RH. The HW treatment was based on previous experiment, in which temperature and durations were tested, and the most effective in reducing CI was 42°C for 15 min. Further experiments were carried out using HW at 42°C for 15 min. One hand represented one replication and 3 replications were used for each treatment. Physical and biochemical changes were determined in the same parameters as the Experiment 1.

Experiment 3: Effects of hot water treatments on expression of *PPO*, *LOX* and *Hsp70* genes in cvs. Hom Thong and Namwa bananas under low temperature storage

Following Experiment 2, the gene expression regulating the browning and the lipid peroxidation enzyme including accumulation of heat shock protein in banana resulted from CI. The gene expression of these enzymes was carried out using semi- quantitative RT-PCR and Real-time PCR technique.

3.1 Total RNA extraction

The protocol was modified from Chang *et al*. (1993) to make it suitable for extracting RNA from banana peel samples. Approximately 3 g of tissue was ground by Mixer Mill (MM 301, Retsch, Germany) under liquid nitrogen. Ground tissue was added to 15 ml of extraction buffer previously warmed to 65°C for 10 min containing 2% hexadecyltrimethyl ammonium bromide (CTAB), 2% polyvinylpyrrolidinone (PVP), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 300 µl of β-mercaptoethanol and 7.5 µl of spermidine in the RNAse-free Falcon tubes, and the contents vortex until well mixed. The mixture was homogenized using a Polytron PT 2100 (Kinematica AG, Switzerland, 1.2 mm diameter head) at speed 12 for 1 min or until the sample foams reached the top of the tube. Fifteen milliliters of chloroform:isoamyl alcohol (24:1, v/v) was added. The mixture vortexed and centrifuged at 7,000 x g for 15 min (4°C). Aqueous phase has filtered through autoclaved Mira cloth into a new RNAse-free Falcon tube, an equal volume of chloroform:isoamyl alcohol added, the solution mixed and centrifuged at 7,000 x g for 15 min (4°C). The top aqueous phase was again transferred to an RNAse-free Oakridge tube and the volume recorded. RNA was precipitated with 8 M lithium chloride (LiCl) to give a final concentration of 2M LiCl in the solution and leaved at 4°C overnight. After centrifuged at 20,000 x g for 15 min at 4°C, the supernatant was carefully poured off and the RNA pellet was washed with 500 µl SSTE buffet containing 1 M NaCl, 0.5% SDS, 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0) and transferred to an RNAse-free eppendorf tube. It was extracted once using 500 µl chloroform:isoamyl alcohol then microcentrifuge at 12,000 x g for 10 min at 4°C . The top aqueous phase was collected and two volumes of ethanol were added, The RNA was precipitated at –70°C for 1 h. The mixture was centrifuged at 12,000 x g for 20 min at 4°C and discarded the supernatant. The pellet was dried and resuspended in 50 µl of cool DEPC-water. After that RNA was quantified by measuring the absorbance at 260 nm. One absorbance unit at 260 nm corresponded to approximately 40 µg /ml (Sambrook and Russel, 2001). The RNA concentration of each sample was estimate in µg/ml by the following equation: RNA concentration = A_{260} x dilution factor

x 40 μg/ml. Quality of the purified total RNA was determined by calculating the A_{260}/A_{280} ratio. The ratio between 1.8-2.0 indicated good quality of the RNA and qualitatively checked using gel electrophoresis on a 0.8%.

3.2 RT-PCR

Five micrograms of total RNA was pretreated with deoxyribonucleaseI (DNaseI, Rnase-free, Fermentas, Canada) to remove contaminating genomic DNA, then used to make cDNA using M-MLV reverse transcriptase (Promega, USA) for lipoxygenase (*LOX*), polyphenol oxidase (*PPO*), heat shock protein 70 (*Hsp70*) and *18S rRNA* genes as described by the supplier's instructions. The synthesized cDNA was used as a template to amplify the target genes by PCR. Specific primers were designed using the Primer 3 program to the conserved amino acid sequences of PPO from accession numbers AY596268, AY149882, DQ532392 and AY866432; LOX from accession numbers EF215449, AJ418043, AY093104 and AF019614; Hsp70 from accession numbers X54030, X67711, X73472 and Z32537; 18S rRNA from accession numbers AF 069226. Sequences of the primers are described in Table 1. The amplification reactions were initially denatured at 94°C for 5 min and then subjected to 35 cycles at the following condition: 94°C for 30s, 55°C for 30 s (*PPO*) or 53°C for 30 s (*LOX* and *Hsp70*) or 50°C for 30 s (*18S rRNA*), and 72°C for 30 s (*PPO, LOX, Hsp70* and 18S rRNA) with a final extension at 72°C for 10 min.

Table 1 Gene and sequences of primers used in RT-PCR analysis

Gene	Forward primer	Reverse primer	Tm (°C)	Expected size (bp)
PPO	5'-TCCACAACTCCTGGCTCTTC-3'	5'-TAGGGTCGGTTCCGTTGTAG-3'	55	243
LOX	5'-AGGAGTTTCCTCCGGTTAGC-3'	5'TCAGAGTGCCATCATCCTTG-3'	53	246
Hsp70	5'-TGAGGAGCTCAACATGGACCTG-3'	5'-AGGTCCTGCACCTTCTCATTGC-3'	53	267
18S rRNA	5'- GTTGCAGTTAAAAAGCTCGT-3'	5'-CCGAAATCCTGTGATGTTAT-3'	50	216

3.3 PCR products cloning and DNA sequencing

The amplified cDNA fragments from each gene were purified using a gel extraction kit (QIAquick® Gel Extraction, Qiagen, Germany) and cloned into pGEM-T vector (pGEM®-T Easy Vector Systems, Promega, USA) as described in the supplier's instructions. The ligations were set up using 5 µl of 2x Rapid Ligation Buffer, 1 µl of pGEM-T vector (50 ng), 3 µl of PCR product, 1 µl of 10x T4 DNA. Ligase and adjusted with deionized water to a final volume of 10 µl. The reactions were mixed by pipetting and then incubated overnight at 4°C.

E. coli DH5-α competent cell was used for the transformations. Two LB/ampicillin/IPTG/X-Gal plates for each ligation reaction were prepared. The ligation reaction (10 µl) was carefully put into 100 µl of frozen DH5-α tube then gently flicked to mix and placed on ice for 20 min. The cells were heat shocked for 45s in a water bath at 42°C and immediately return on ice for 2 min. Then 1 ml of SOC medium was added to the tube and incubated for 1 h at 37°C with shaking approximately 150 rpm. Each transformation culture (100 ml) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37°C. Single white colonies would be selected for the next step.

3.4 Purification of plasmid DNA using a miniprep kit (QIAprep® Spin Miniprep Kit, Quaigen, Germany).

Single white colonies containing the vector and inserted genes were cultured in 5 ml of LB medium overnight. Cells were centrifuged at 14,000 x g for 3 min and collected the pellet cells. The pellet cells were re-suspended in 250 µl of re-suspension buffer and transferred to 1.5 ml microtube. Then, 250 µl of lysis buffer was added and the tube gently inverted to mix. Three hundred and fifty µl of neutralization buffer were added and inverted immediately then centrifuged at 14,000 x g for 10 min. The supernatant was transferred to the spin column then centrifuged for 1 min and the flow-through discarded. The spin column was washed by adding 750 µl of washing buffer and centrifuged again for 1 min, the-flow-through discarded and centrifuged to

remove the residual washing buffer. DNA was eluted by adding 50 µl of elution buffer and centrifuging for 1 min. DNA was kept at -20°C until used for cutting with the ECoRI restriction enzyme (New England Biolabs, Inc., USA) and the insert size was checked by gel electrophoresis before sequencing.

3.5 DNA sequencing and analysis

The sequence analysis was conducted by automatic sequencing using the ABI PRISM® 377 DNA sequencer (Applied Biosystems, USA). The sequences of amplified *PPO, LOX, HSP70* and *18S rRNA* fragments were compared with the genes in the Genbank database using the BLAST program from NCBI (National Centre for Biotechnology Information).

3.6 Analysis of gene expression by semi-quantitative RT-PCR

Semi-quantitative RT-PCR was conducted for studying the pattern of gene expression. To find the suitable cycle of thermocycler for normalization of *Musa* cDNA, 18S rRNA primer was used as the reference gene. All specific primers for semi-quantitative RT-PCR was designed based on the ABI supplier's instruction and set to the annealing temperature 50-55°C with the amplication size of 200-250 bp. In the experiments, tested a number of cycles ranging from 24 to 36 and selected the suitable conditions. Reaction of semi-quantitative RT-PCR of each gene, i.c. *PPO* were subjected to one cycle of 94°C for 5 min, 20 cycles (94°C for 30 s, 55°C for 30 s and 72°C for 30 s) and one cycle of 72°C for 10 min; *LOX* and *Hsp70* were subjected to one cycle of 94°C for 5 min, 24 cycles (94°C for 30 s, 53°C for 30 s and 72°C for 30 s) and one cycle of 72°C for 10 min; 18S rRNA were subjected to one cycle of 94°C for 5 min, 18 cycles (94°C for 30 s, 50°C for 30 s and 72°C for 30 s) and one cycle of 72°C for 10 min. The reaction components were used for semi-quantitative RT-PCR as follows:

10x PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
10 µM dNTPs	0.5 µl
10 µM forward primer	1.0 µl
10 µM reverse primer	1.0 µl
Taq polymerase enzyme	0.25 µl
cDNA template	2.0 µl
Distilled water	15.25 µl
Total	25 µl

The reaction product was separated with 1.2% agarose gel electrophoresis at 100 volts for 30 min. The gel was stained in 2.5 µg/ml ethidium bromide (EtBr) solution for 15 min. The RNA bands were visualized under UV transilluminator and photographed by SYNGENE BIO IMAGINE Gel Documentation (Syngene, England).

3.7 Quantitative real-time PCR

To confirm results of gene expression, the real-time PCR analyses of reference and target gene transcripts in cDNA samples were conducted in an ABI model 7000 real-time PCR machine (Applied Biosystems, USA). A 25 µl PCR reaction was prepared containing 2 µl of cDNA template, 12.5 µl of SYBR® Green PCR master mix (QuantiTect™ SYBR® Green PCR kit, Qiagen, Germany), 1 µl of each primer and 8.5 µl of Distilled water. The primers of each gene were used the same condition as above. Direct detection of the PCR product was measured by monitoring the increased in fluorescence caused by the binding of SYBR® Green dye to double stranded DNA. A fluorescence threshold was set manually to ΔRn on the log fluorescence scale to

determine the fractional cycle number (Ct value) at which the fluorescence passed the detection threshold. For each cDNA samples, relative expression levels of each protein coding gene were normalization by reference to the rRNA gene assay. The transcript abundance ratio of target gene to reference gene was analyzed by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and presented as the relative value of gene expression. Formular of relative gene expression is $2^{-\Delta\Delta Ct}$

$\Delta\ \Delta Ct = (C_{t\ Target} - C_{t\ reference})_{\ Timex} - (C_{t\ Target} - C_{t\ reference})_{\ Time0}$

Where: Ct is the number of PCR cycle

Time x is any time point

Time 0 represents the 1x expression of the target gene normalized to *18s rRNA*

The Experimental Time

The experiments were conducted during April 2004-June 2007.

The Experimental Places

1. Banana orchards in Petchaburi Province, Thailand.
2. Postharvest Research Unit, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand.
3. Mann Laboratory, Department of Plant Sciences, University of California, Davis, CA USA.

RESULTS

Experiment 1: Effect of low temperature on physiological and biochemical changes of two banana cultivars in relation to chilling injury

1.1 CI evaluation

Figure 5 shows the development of peel blackening in banana cvs. Hom Thong and Namwa stored at 4 and 12ºC for 0 to day 10. In fruit stored at 12ºC, slight peel blackening was observed in cv. Hom Thong and became more severe towards the end of the storage period (Figure 6A, Appendix Table 1). Fruit of cv. Hom Thong stored at 4ºC showed drastic blackening by day 2 and reached a maximum blackening by day 10 (Figure 6B, Appendix Table 1). In contrast, fruit of cv. Namwa showed a slight peel blackening on day 4 and remained unchanged until day 10 (Figure 6B, Appendix Table 1).

1.2 Fruit peel color

L values of the peel slightly decreased and were significantly different in both banana cultivars stored at 4ºC (Figure 7B, Appendix Table 2), while L values of the peel stored at 12ºC slowly increased during the first 4 days then remained stable until the end of storage (Figure 7A, Appendix Table 2).

1.3 Membrane permeability

Electrolyte leakage of the peel banana in both cultivars slightly increased and was not significantly different during 8-day storage at 12ºC and electrolyte leakage of cv. Hom Thong increased significantly higher than that in cv. Namwa on day 10 (Figure 8A, Appendix Table 3). Electrolyte leakage of the banana peel of cvs. Hom Thong and Namwa changed slightly during 4-day storage at 4ºC and electrolyte leakage of cv. Hom Thong increased more rapidly than that of cv. Namwa until the end of storage period (Figure 8B, Appendix Table 3).

1.4 TBA-reactive compounds

The levels of TBA-reactive compounds slightly increased in fruit stored at 12ºC. A clear increase of TBA-reactive compounds was observed only in cv. Hom Thong stored at 4°C on day 8 and 10 of storage (Figure 9A, Appendix Table 4), while TBA-reactive compounds of both cvs. Hom Thong and Namwa stored at 4°C started to increase on day 4 to the end of storage periods but TBA-reactive compounds of cv. Hom Thong increased more rapidly than that of cv. Namwa (Figure 9B, Appendix Table 4). A significant difference ($P \leq 0.05$) between two cultivars was only found at 4°C on day 8 and 10.

1.5 Changes in fatty acid composition

Fatty acids found in the peel of both cultivars were palmitic (16:0), stearic (18:0), behenic (22:0), elaidic (18:1), linoleic (18:2) and linolenic (18:3) acids. The proportion of stearic (saturated fatty acid) and elaidic (unsaturated fatty acid) were the lowest whereas the highest were found in the proportion of palmitic (saturated fatty acid) and linoleic (unsaturated fatty acid) (Table 2A). The content of total saturated fatty acid increased in both cultivars during stored at 4ºC and a similar trend also found at 12ºC. At 12ºC, there was no significant change in unsaturated fatty acid during 8 days in both cultivars, while at 4ºC, cv. Namwa had a higher level of unsaturated fatty acid than in cv. Hom Thong. Changes in the ratio of unsaturated to saturated fatty acids [(18:1 + 18:2 + 18:3) / (16:0+18:0+22:0)] stored at 4 and 12ºC were higher in cv. Namwa than in cv. Hom Thong throughout the experiment. By the time of storage at low temperature the ratios had declined, and were similar in both cultivars (Table 2B).

1.6 Total free phenolics

Levels of peel free phenolics in both cultivars at initial time were not significantly different and remained stable during storage at 12 ºC (Figure 10A, Appendix Table 5). Changes in total free phenolics in the peel of both banana cvs. Hom Thong and Namwa

slightly increased to a maximum on day 6 and markedly decreased thereafter during stored at 4 °C. But total phenolics contents in both banana cultivars were not significantly different (Figure 10B, Appendix Table 5).

1.7 LOX activity

Figure 11A and 11B, Appendix Table 6 shows the LOX activity in the peel of both banana cultivars. Little change was found at the higher storage temperature. An increase to a highest level of LOX activity was observed on day 2 in cv. Hom Thong stored at low temperature then slightly decreased thereafter. A gradual increase in LOX activity was also detected in cv. Namwa fruit, but only by day 8 and 10. Except day 0, statistical differences ($P \leq 0.05$) between the cultivars were found at 4°C

1.8 PPO activity

Little change occurred in fruit stored at 12ºC (Figure 12A, Appendix Table 7). An increase in PPO activity was found in cv. Hom Thong during storage at 4ºC. However, in cv. Namwa stored at the low temperature no consistent increase in PPO activity was found. Statistically significant differences ($P \leq 0.05$) between the cultivars were found throughout the experiment at 4°C (Figure 12B, Appendix Table 7).

1.9 POD activity

Figure 13 shows the measured peel POD activities. Initially, only small differences were found between the two cultivars. By day 8 and 10 the activities were higher in cv. Hom Thong, but this occurred at both 12 and 4ºC (Figure 13A and 13B, Appendix Table 8). Statistical differences ($P \leq 0.05$) between the cultivars were found at both 12 and 4°C on day 8 and 10.

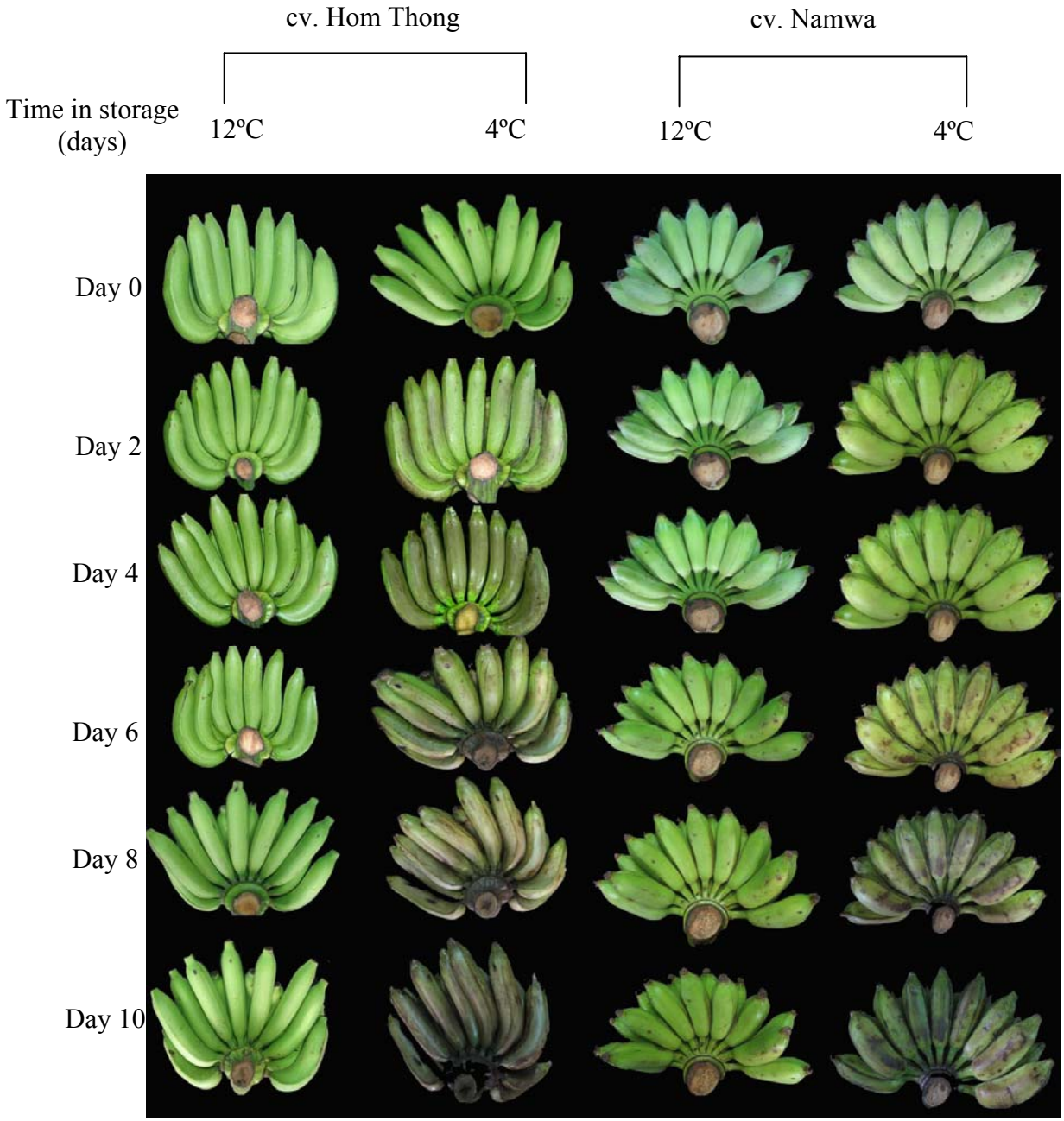

Figure 5 Peel blackening in banana cvs. Hom Thong and Namwa stored at 4 and 12ºC for 10 days.

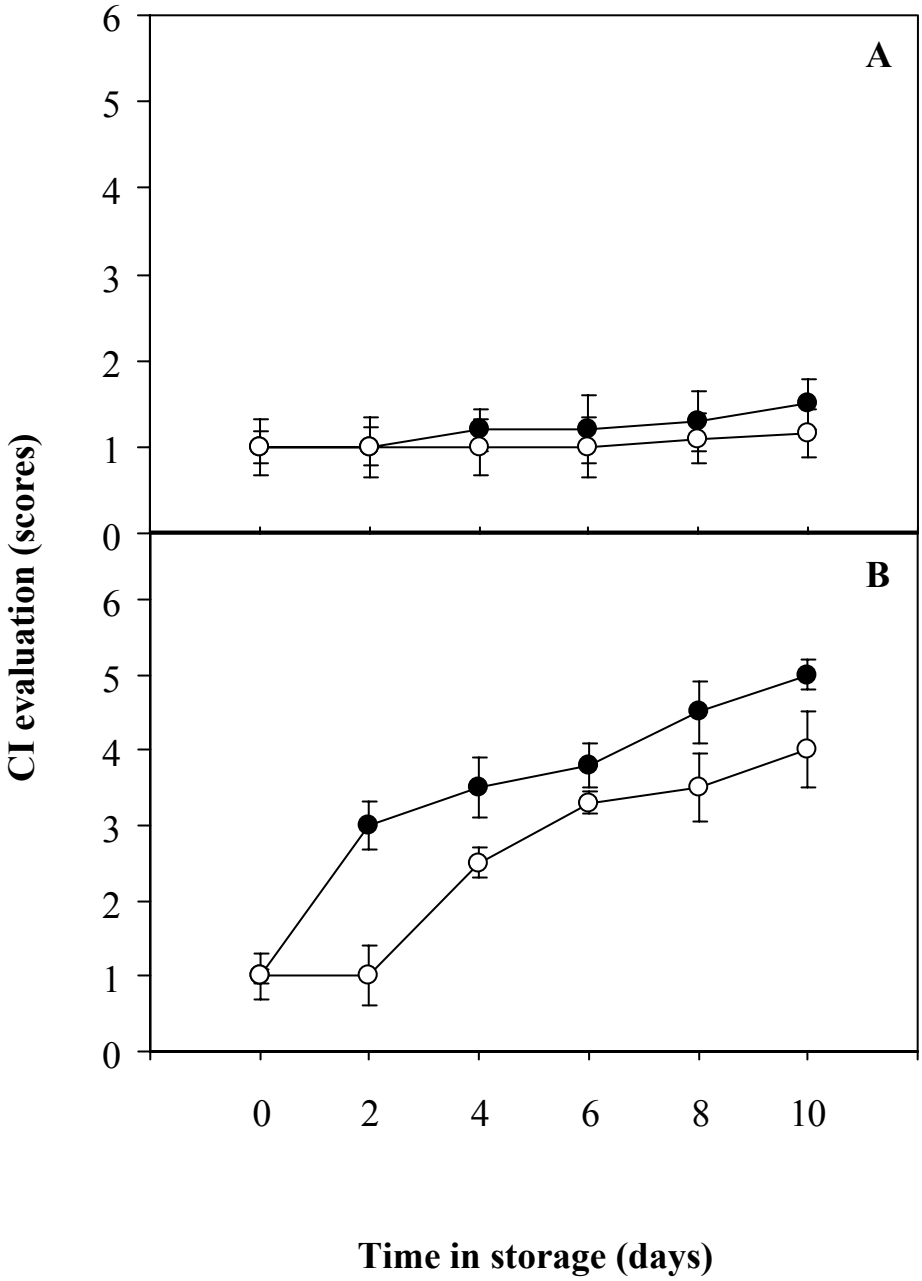

Figure 6 CI evaluations in cvs. Hom Thong (●) and Namwa (○) stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

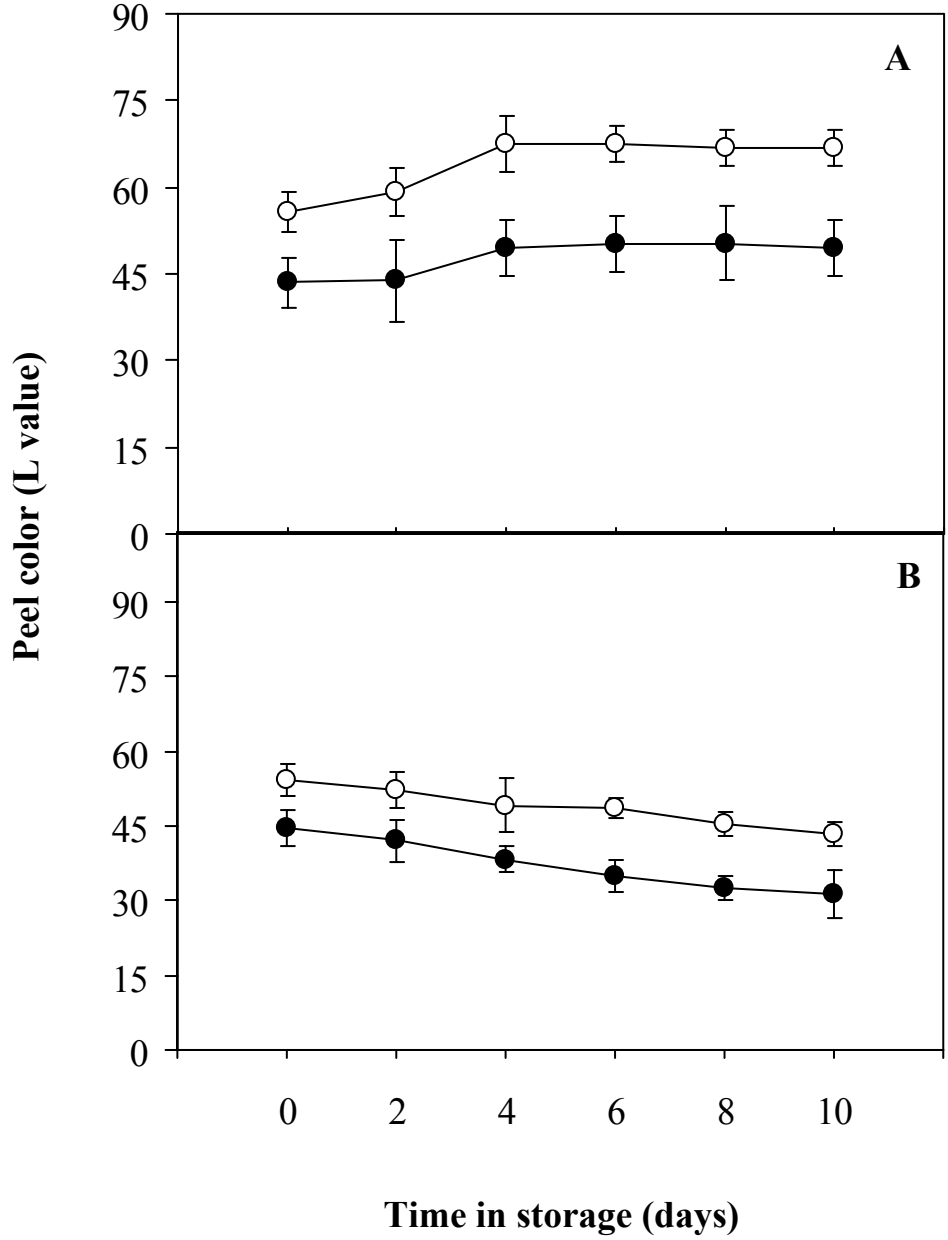

Figure 7 Changes in peel color (L value) of cvs. Hom Thong (●) and Namwa (○) stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

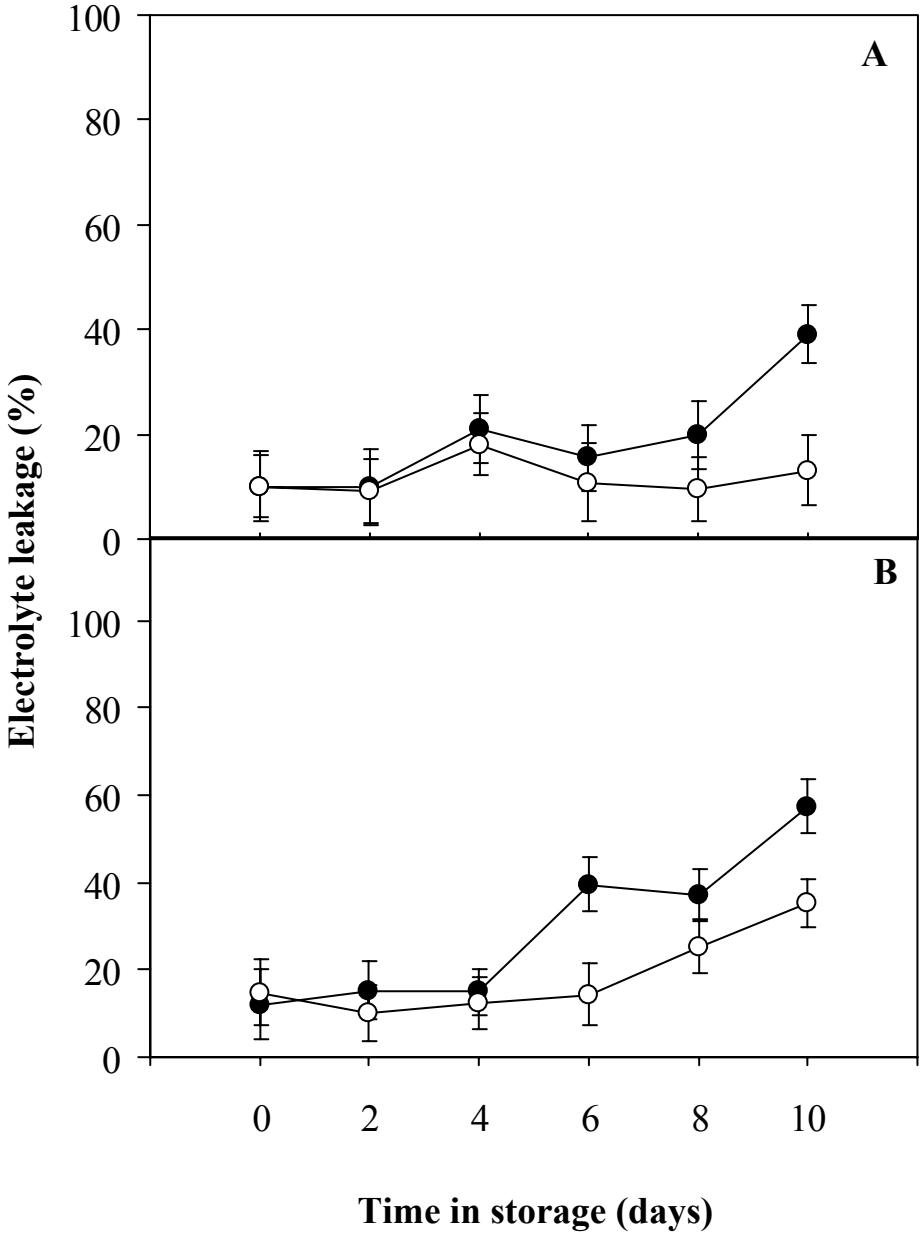

Figure 8 Changes in electrolyte leakage (%) of cvs. Hom Thong (●) and Namwa (○) banana peel stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

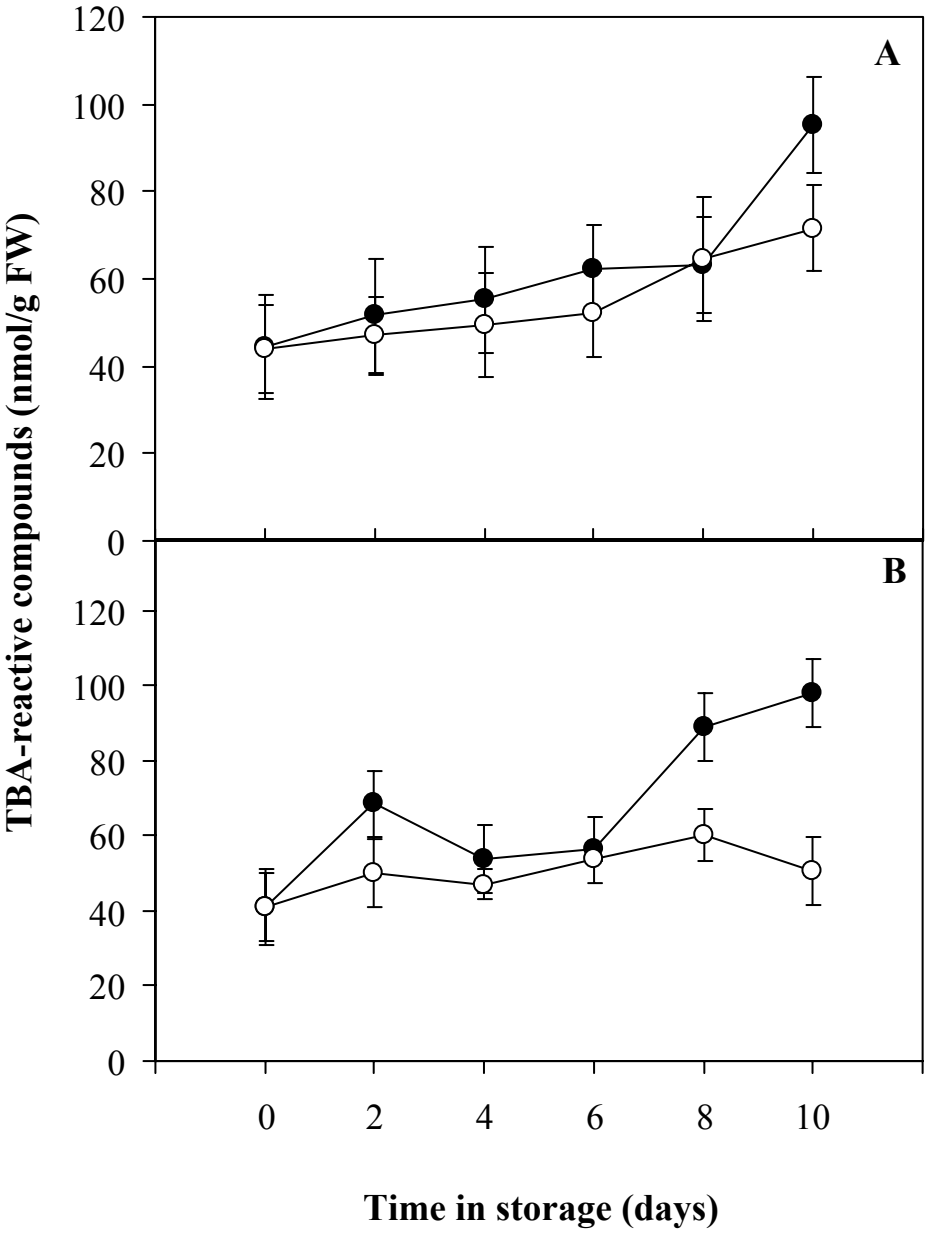

Figure 9 Changes in level of TBA-reactive compounds of cvs. Hom Thong (●) and Namwa (○) banana peel stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

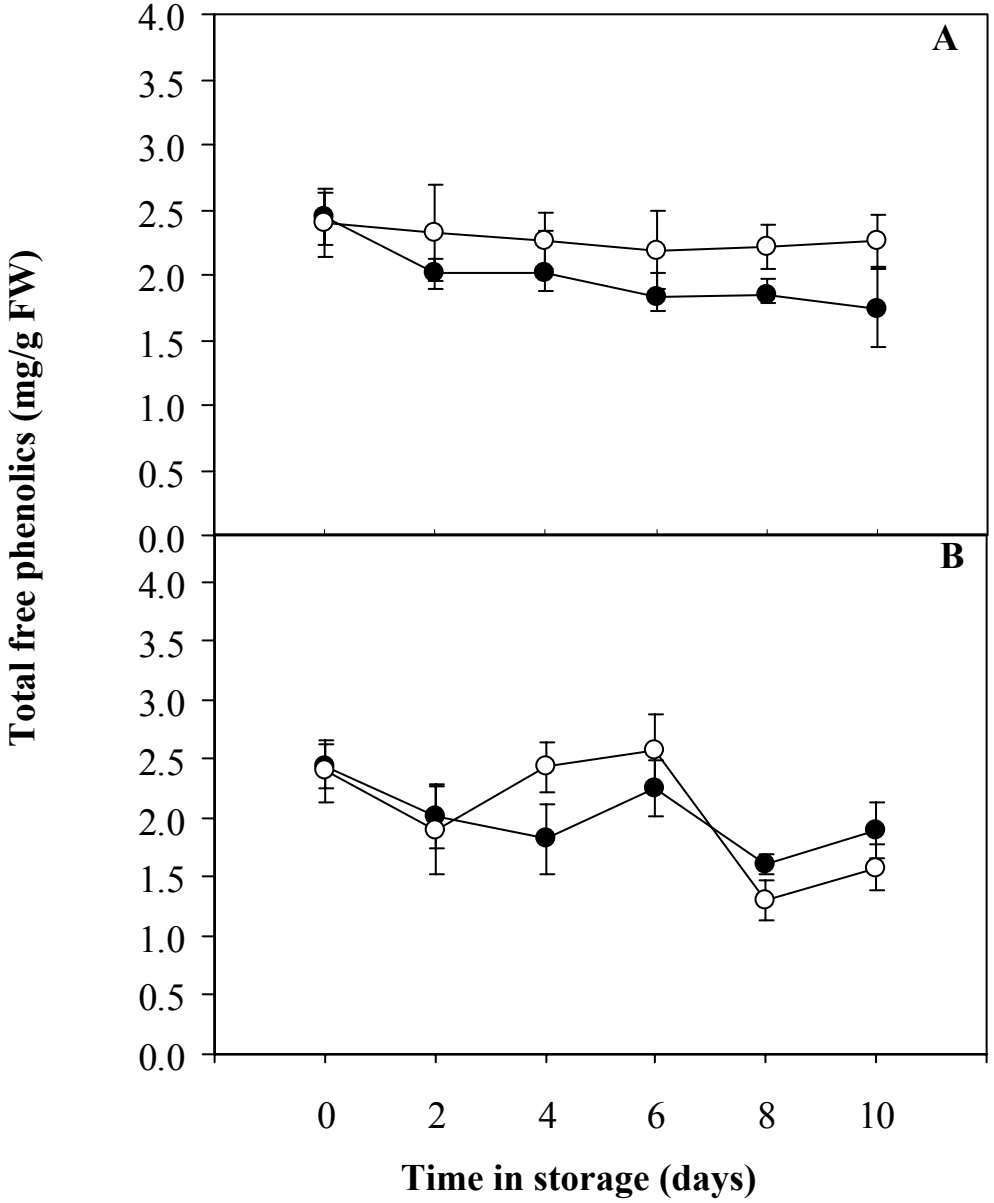

Figure 10 Changes in total free phenolics of cvs. Hom Thong (●) and Namwa (○) banana peel stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

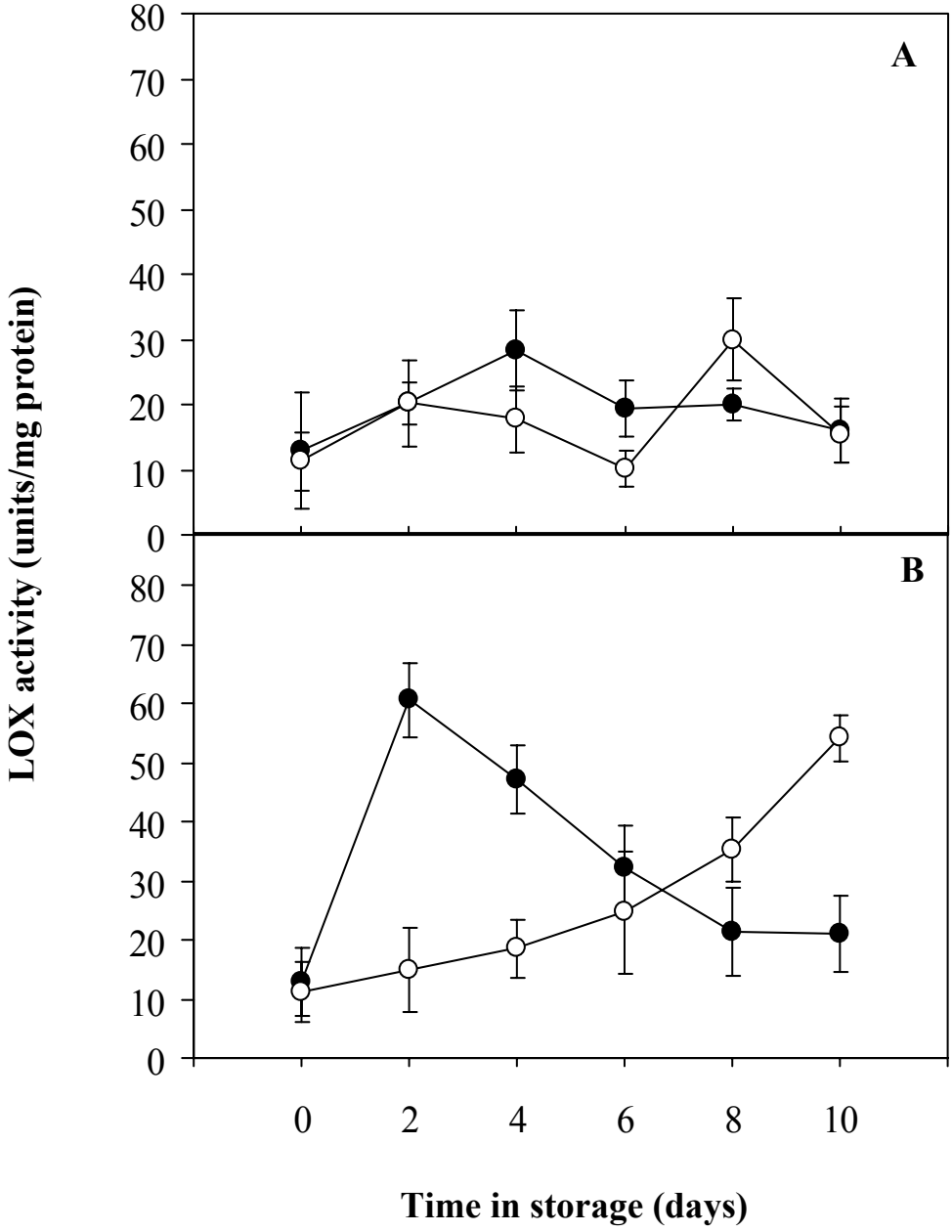

Figure 11 Changes in LOX activities of cvs. Hom Thong (●) and Namwa (○) banana peel stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

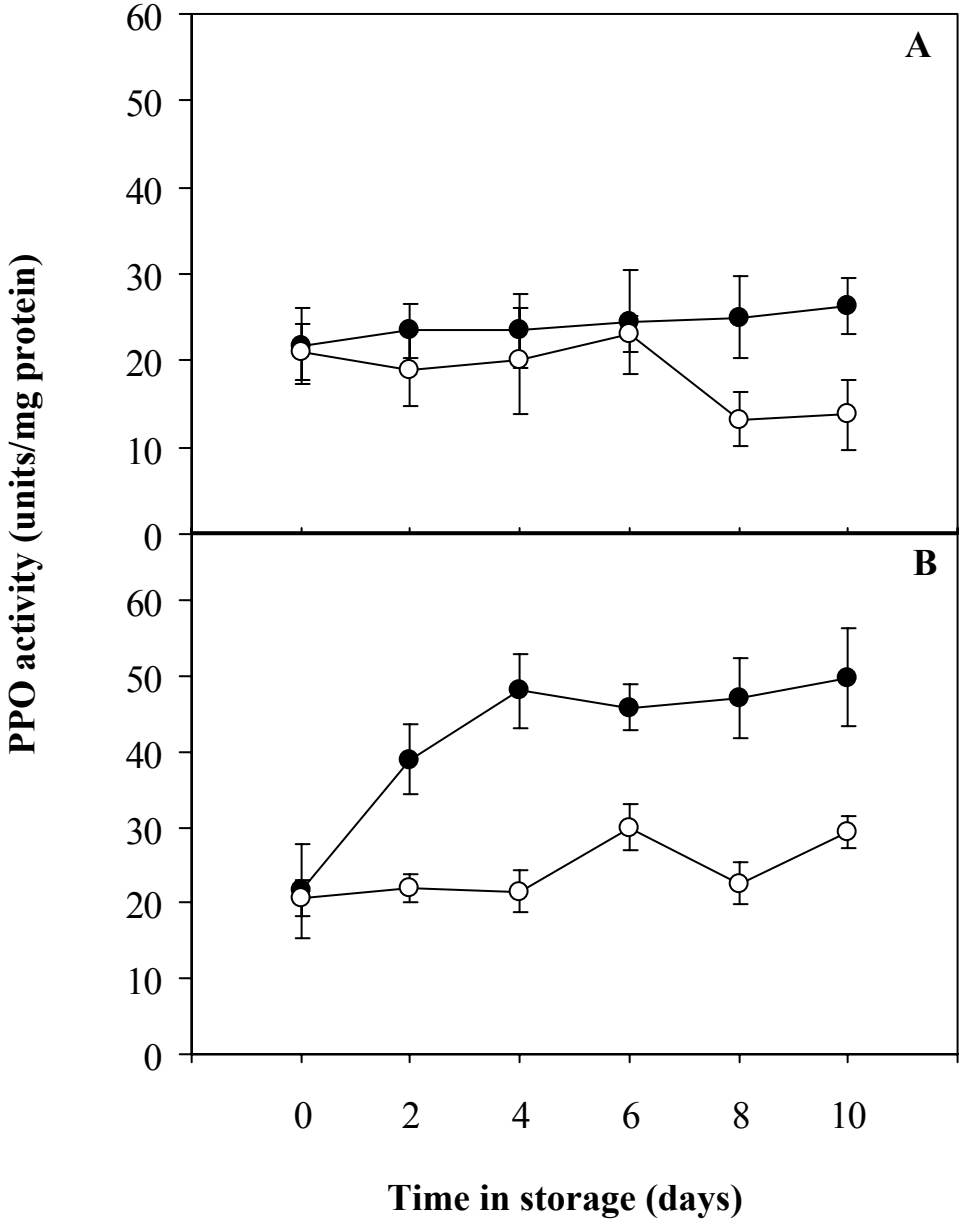

Figure 12 Changes in PPO activities of cvs. Hom Thong (●) and Namwa (○) banana peel stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

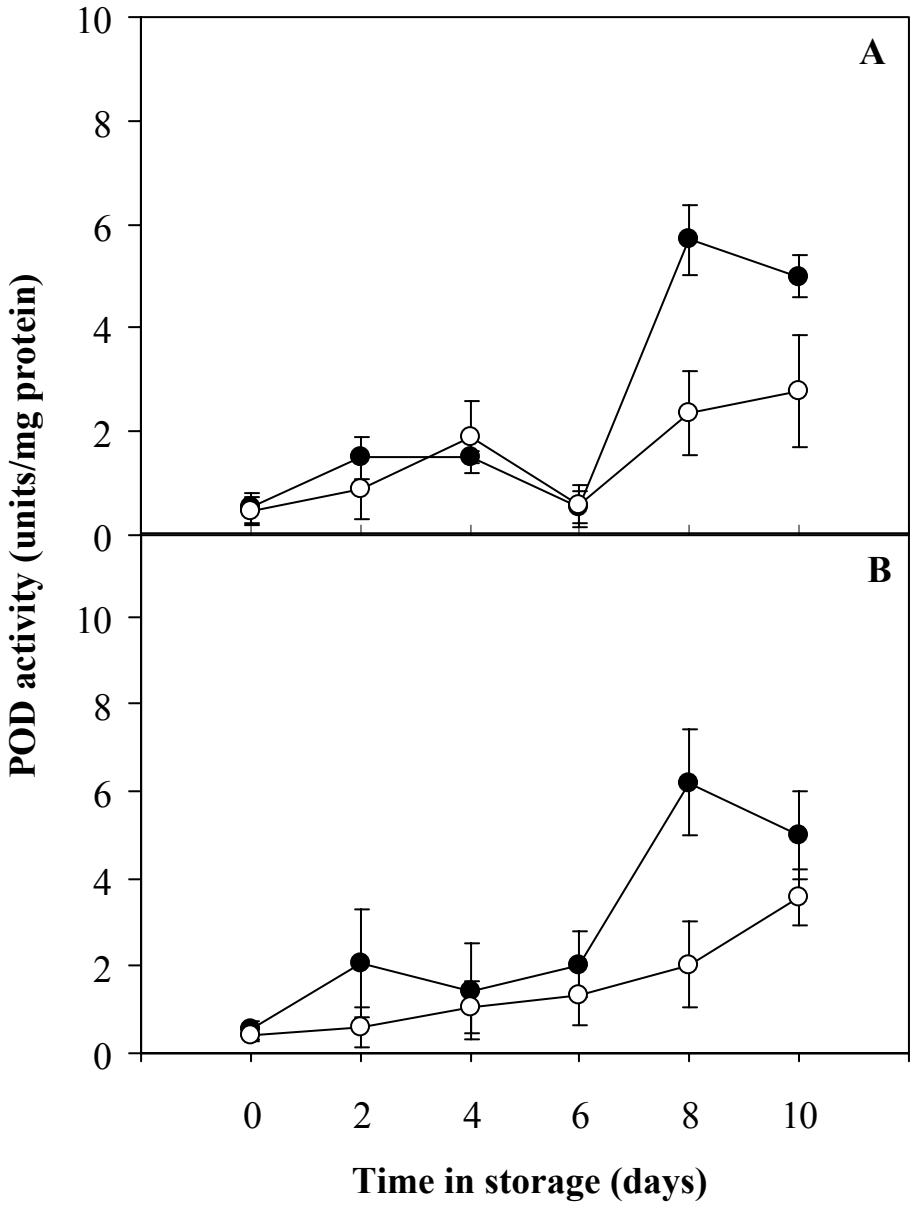

Figure 13 Changes in POD activities of cvs. Hom Thong (●) and Namwa (○) banana peel stored at 12 (A) and 4ºC (B). Data are means ± SE of three replications.

Table 2A Fatty acid composition (g /100 g fatty acid) in the peel of cvs. Hom Thong and Namwa stored at 4 and 12°C.

Treatments	Time in storage (day)	Saturated fatty acids			Unsaturated fatty acids		
		Palmitic acid (16:0)	Stearic acid (18:0)	Beheric acid (22:0)	Elaidic acid (18:1, trans)	Linoleic acid (18:2, cis)	Linolenic acid (18:3)
cv.Hom Thong stored at 12ºC	0	38.80b	4.30b	7.19d	7.32a	27.91b	9.30b
	4	38.25b	4.68b	9.78c	6.42b	26.00b	7.79c
	8	38.38b	4.71b	10.02c	6.21b	25.95b	7.56c
cv. Hom Thong stored at 4 ºC	0	38.89b	4.38b	7.28d	7.37a	27.95b	9.00b
	4	43.73a	4.15b	8.17d	6.68b	24.18c	6.80c
	8	43.39a	4.23b	9.03c	6.55b	22.09d	6.64c
cv.Namwa stored at 12ºC	0	33.45c	4.50b	6.00e	6.32b	30.42a	15.30a
	4	38.33b	4.00b	17.13b	5.21b	26.74b	17.80a
	8	39.00b	4.11b	20.08a	5.56b	24.00c	17.67a
cv. Namwa stored at 4ºC	0	33.35c	4.53b	5.99e	6.36b	30.53a	15.37a
	4	42.64a	5.22a	8.98d	5.00b	24.33c	11.98b
	8	41.26a	5.73a	9.06c	5.22b	23.11d	12.01b

Data are average of 3 biological replications.

Within each column a different letter shows statistical difference at $P \leq 0.05$.

Table 2B Concentrations (g /100 g fatty acid) of unsaturated fatty acids (UFA), saturated fatty acids (SFA) and their ratio in the peel of cvs. Hom Thong and Namwa stored at 4 and 12°C.

Treatments	Time in storage (day)	UFA	SFA	UFA/SFA
cv. Hom Thong stored at 12ºC	0	44.53b	50.29d	0.88b
	4	40.21b	52.71d	0.76b
	8	39.72b	53.11d	0.75b
cv. Hom Thong stored at 4ºC	0	44.32b	50.55d	0.88b
	4	37.66c	56.05c	0.67c
	8	35.28c	56.65c	0.62c
cv. Namwa stored at 12ºC	0	52.04a	43.95e	1.18a
	4	49.75a	59.46b	0.84b
	8	47.23a	63.19a	0.75b
cv. Namwa stored at 4ºC	0	52.26a	43.87e	1.19a
	4	41.31b	56.84c	0.73a
	8	40.34b	56.05c	0.72a

Data are average of 3 biological replications.

Within each column a different letter shows statistical difference at $P \leq 0.05$.

Experiment: 2 Effect of hot water treatments (HW) on reducing CI of banana during low temperature storage

2.1 CI evaluation

Figure 14 shows the development of peel blackening in cvs. Hom Thong and Namwa banana with and without HW prior to storage at 4 ºC for 10 days. Fruit of cv. Hom Thong without HW (control) treatment prior to storage at 4°C already showed peel blackening on day 2 and continuously reached a maximum blackening on day 10 (Figure 15A, Appendix Table 9), while fruit of cv. Namwa became blackening on day 4 (Figure 15B, Appendix Table 9). These results confirmed the results of Experiment1. Hands of both cultivars were immersed in hot water at 42 ºC for 5, 10 and 15 min. HW treatment for 5 or 10 min had no effect on CI, while HW treatment for 15 min delayed the time to peel blackening and significantly reduced CI in cv. Hom Thong by about 4 days and in cv. Namwa by about 2 days (Figure 15A and 15B, Appendix Table 9).

2.2 Fruit peel color

L values of the peel slightly decreased in cv. Hom Thong with and without HW throughout the storage period at 4 ºC and significantly different found on day 4 and day 6. While in cv. Namwa, L values of the peel remained stable in both with and without HW until the end of storage (Figure 16, Appendix Table 10).

2.3 Membrane permeability

As regard to membrane permeability, electrolyte leakage of the peel in cv. Hom Thong without HW slightly increased during the first 6 days then increased rapidly in the last 4 days. While electrolyte leakage of cv. Hom Thong with HW remained stable and started to slightly increase after 4 days and the levels of electrolyte leakage were lower than in cv. Hom Thong without HW. The lowest level of electrolyte leakage was found in the peel of cv. Namwa with and without HW. It

showed a slight increase between day 6 and day 10 and there was no significant difference ($P \leq 0.05$) in cv. Namwa between with and without HW treatment (Figure 17, Appendix Table 11).

2.4 TBA-reactive compounds and LOX activity

The TBA- reactive compound levels (Figure 18A, Appendix Table 12) in cv. Hom Thong were lower after the HW treatment at 42 ºC for 15 min than that in control, whereas no differences were detectable in cv. Namwa. Similarly, the activities of LOX in cv. Hom Thong were reduced at different levels after HW treatment while in cv. Namwa was similar between HW treatment and control. LOX activity was maintained constantly during 8 days and slightly increased toward the end of storage time (Figure 18B, Appendix Table 13).

2.5 Fatty acid composition

Fatty acids found in the peel of both cultivars were the same as in Experiment 1. There were palmitic (16:0), stearic (18:0), behenic (22:0), elaidic (18:1), linoleic (18:2) and linolenic (18:3) acids. Among the saturated fatty acids (SFA), palmitic acid was predominant, while linoleic acid was the major unsaturated fatty acid (UFA) (Table 3A). During storage time, the total levels of UFA (expressed as grams per 100 gram fatty acid) in both cultivars with HW treatment were higher than in control. During the first 8 days of stored at 4ºC, the ratios of UFA/SFA [(18:1 + 18:2 + 18:3) / (16:0+18:0+22:0)] increased in HW treatment while a slight decrease was found in control and they were similar in both cultivars. The increase in the ratios of UFA/SFA was higher in cv. Namwa than in cv. Hom Thong (Table 3B).

2.6 Total free phenolics

During storage at 4ºC, levels of free phenolics in the peel were similar in both cultivars and HW treatment had no effect (Figure 19, Appendix Table 13). In cv. Hom Thong, total free phenolics contents slowly decreased on day 2 and increased to

a maximum on day 4 then decreased again until the end of the experiment but there was no significant difference ($P \leq 0.05$) between the treatment with and without HW. The total free phenolics contents in cv. Namwa with and without HW slightly decreased until day 6 and remained stable until the end of storage time (Figure 19, Appendix Table 14).

2.7 PPO activity

Changes in PPO activities in the peel are shown in Figure 20 and Appendix Table 15. An increase in PPO activity was found only in cv. Hom Thong without heat treatment. In both cultivars, the PPO activities were considerably lower from day 2 in cv. Hom Thong and from day 6 in cv. Namwa after the HW treatment, compared with no such treatment.

2.8 POD activity

Changes of POD activity in the peel of cv. Hom Thong without HW treatment was much higher than that of banana with HW. It increased sharply after day 4 and then remained unchanged. While in cv. Hom Thong with HW treatment, the activity of POD continuously declined during day 2 and day 8 then slightly increased at the end of storage. The activity of POD in cv. Namwa with and without HW treatment remained stable throughout the storage time (Figure 21, Appendix Table 16).

Table 3A Fatty acid composition (g /100 g fatty acid) in banana peel. Fruit of cvs. Hom Thong and Namwa were stored at 4 °C, with or without prior immersion in hot water (42 °C for 15 min).

Treatments	Days at 4 °C	Saturated fatty acids			Unsaturated fatty acids		
		Palmitic acid (16:0)	Stearic acid (18:0)	Beheric acid (22:0)	Elaidic acid (18:1, trans)	Linoleic acid (18:2, cis)	Linolenic acid (18:3)
cv. Hom Thong Control	0	52.21 ab	5.11 b	5.28 c	6.62 b	25.21 c	10.1 d
	4	55.64 a	7.32 a	8.76 b	6.01 b	24.69 c	7.76 e
	8	55.02 a	7.08 a	8.09 b	7.11 b	23.05 c	7.29 e
cv. Hom Thong Hot water	0	49.05 b	5.21 b	10.02 a	6.01 b	24.21 c	12.10 c
	4	48.25 b	4.66 b	10.12 a	6.51 b	23.77 c	14.50 c
	8	48.51 b	4.96 b	7.77 b	6.66 b	30.01 b	9.44 d
cv. Namwa Control	0	38.28 c	5.19 b	5.02 c	5.90 b	27.02 b	19.51 a
	4	51.08 ab	6.91 a	5.11 c	9.82 a	29.09 b	19.96 a
	8	51.19 ab	6.88 a	5.08 c	10.51 a	29.58 b	20.50 a
cv. Namwa Hot water	0	38.01 c	4.25 b	6.61 b	9.46 a	30.55 b	16.09 b
	4	37.77 c	4.33 b	5.34 c	10.66 a	36.82 a	15.83 b
	8	34.00 d	4.16 b	5.44 c	11.17 a	36.99 a	15.69 b

Within each column a different letter shows statistical difference at $P \leq 0.05$.

Table 3B Concentrations (g /100 g fatty acid) of unsaturated fatty acids (UFA), saturated fatty acids (SFA) and their ratio in banana peel. Fruit of the cvs. Hom Thong and Namwa were stored at 4 ℃, with or without prior immersion in hot water (42 ℃ for 15 min).

Treatments	Days at 4 ℃	UFA	SFA	UFA/SFA
cv. Hom Thong Control	0	41.93 d	62.60 b	0.67 d
	4	38.46 e	71.72 a	0.54 e
	8	37.45 e	70.19 a	0.53 e
cv. Hom Thong Hot water	0	42.32 d	64.28 b	0.66 d
	4	44.78 c	63.03 b	0.71 c
	8	46.11 c	61.24 b	0.75 c
cv. Namwa Control	0	52.43 b	48.49 c	1.08 b
	4	58.87 ab	63.10 b	0.93 b
	8	60.59 a	63.15 b	0.96 b
cv. Namwa Hot water	0	56.10 b	48.87 c	1.15 b
	4	63.31 a	47.44 c	1.33 a
	8	63.85 a	43.60 c	1.46 a

Within each column a different letter shows statistical difference at $P \leq 0.05$.

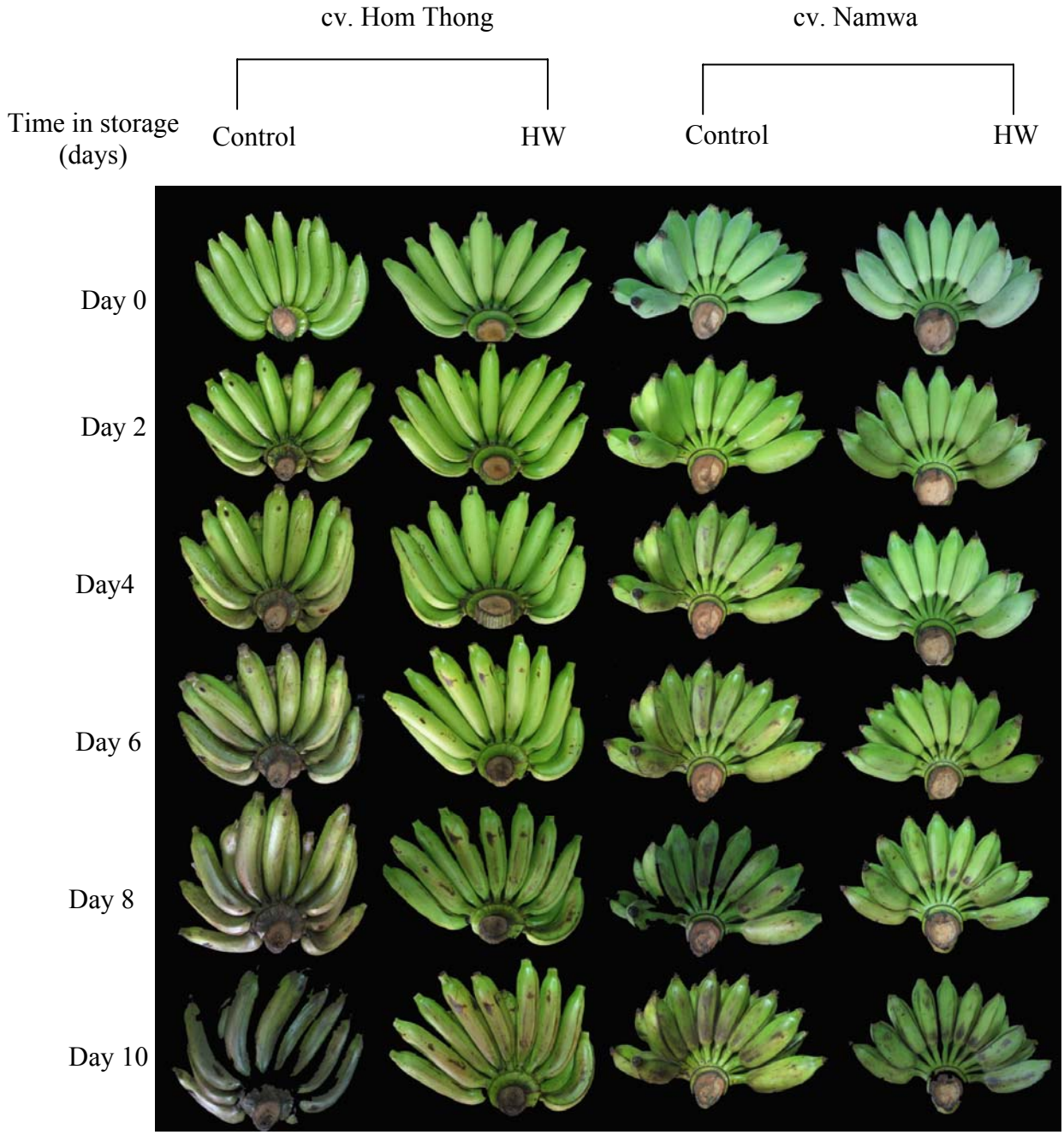

Figure 14 Peel blackening in cvs. Hom Thong and Namwa with and without HW stored at 4ºC for 10 days.

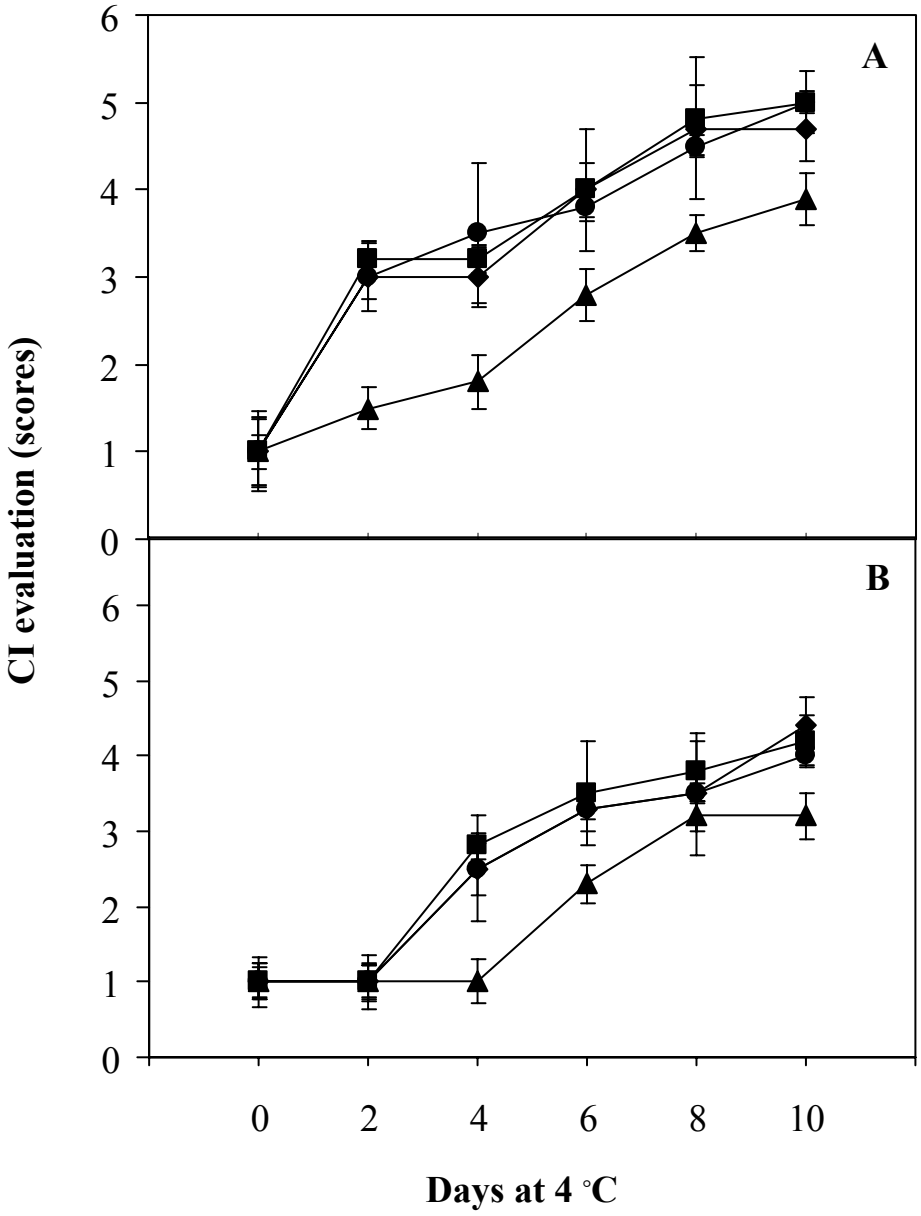

Figure 15 CI evaluation in cvs. Hom Thong (A) and Namwa (B) stored at 4ºC without (●) and with HW at 42ºC for 5 (■), 10 (◆) and 15 (▲) min. Data are means ± SE of three replications.

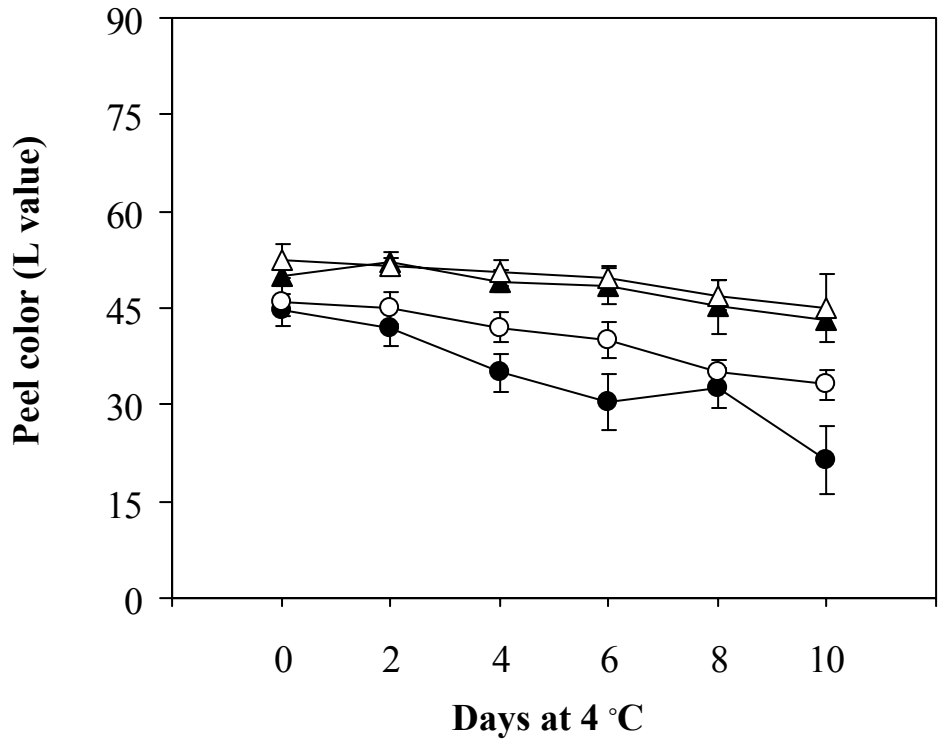

Figure 16 Changes in peel color (L value) of cv. Hom Thong banana peel with (○) and without (●) HW at 42ºC for 15 min and cv. Namwa banana peel with (△) and without (▲) HW stored at 4ºC. Data are means ± SE of three replications.

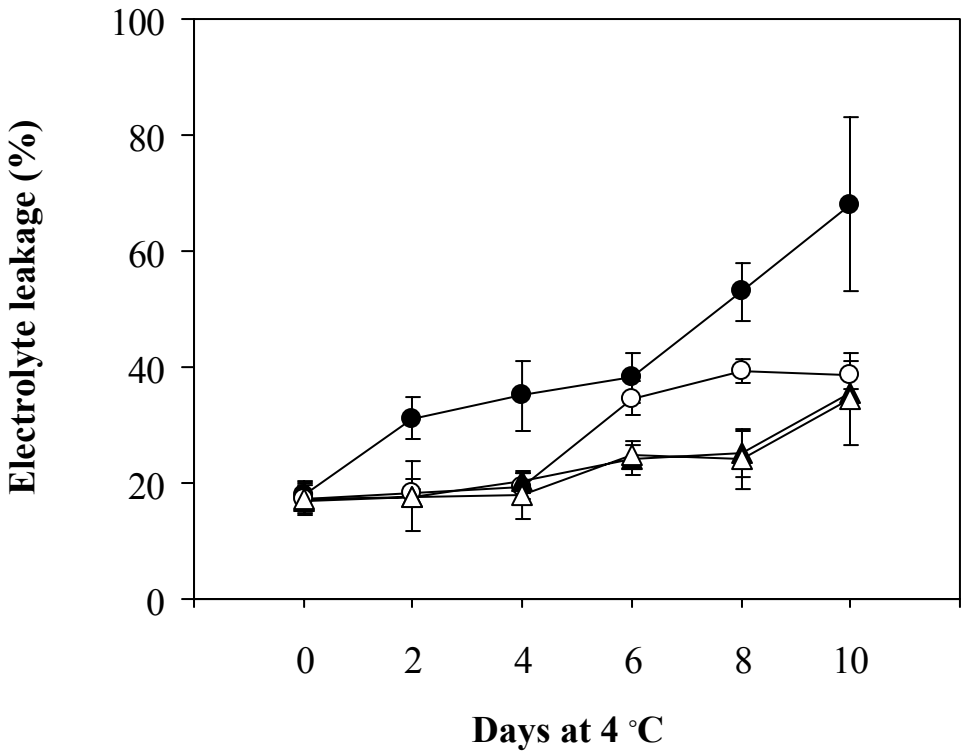

Figure17 Changes in electrolyte leakage (%) of cv. Hom Thong banana peel with (○) and without (●) HW at 42ºC for 15 min and cv. Namwa banana peel with (△) and without (▲) HW stored at 4ºC. Data are means ± SE of three replications.

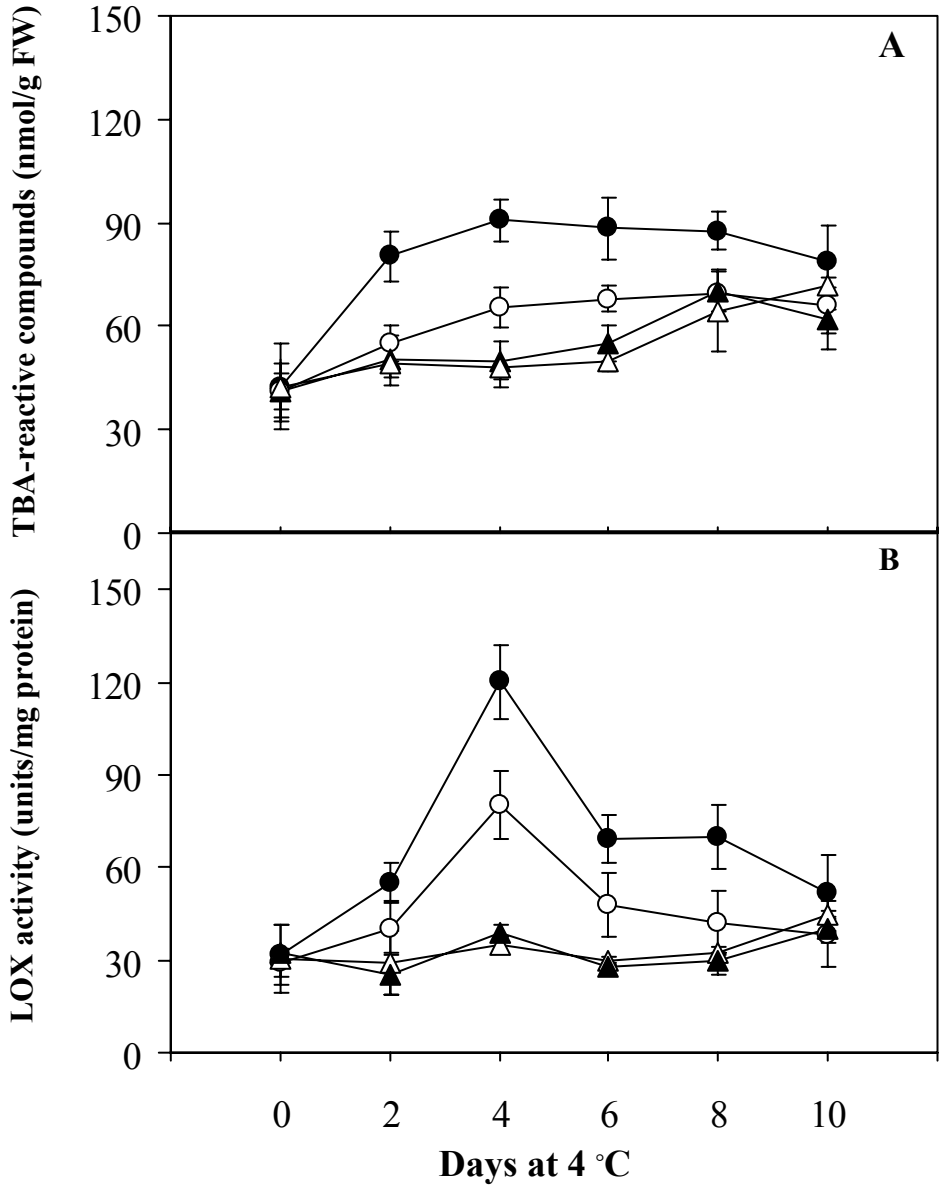

Figure 18 Changes in level of TBA-reactive compounds (A) and LOX activities (B) of cv. Hom Thong banana peel with (○) and without (●) HW at 42 ºC for 15 min and cv. Namwa banana peel with (△) and without (▲) HW stored at 4 ºC. Data are means ± SE of three replications.

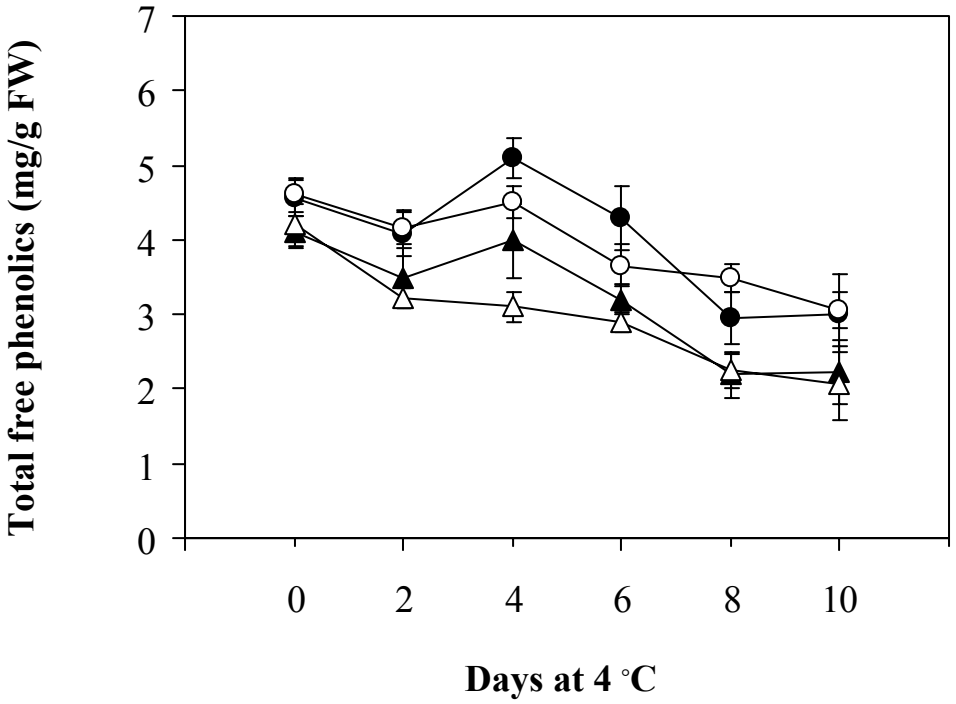

Figure 19 Changes in total phenolics of cv. Hom Thong banana peel with (○) and without (●) HW at 42ºC for 15 min and cv. Namwa banana peel with (△) and without (▲) HW stored at 4ºC. Data are means ± SE of three replications.

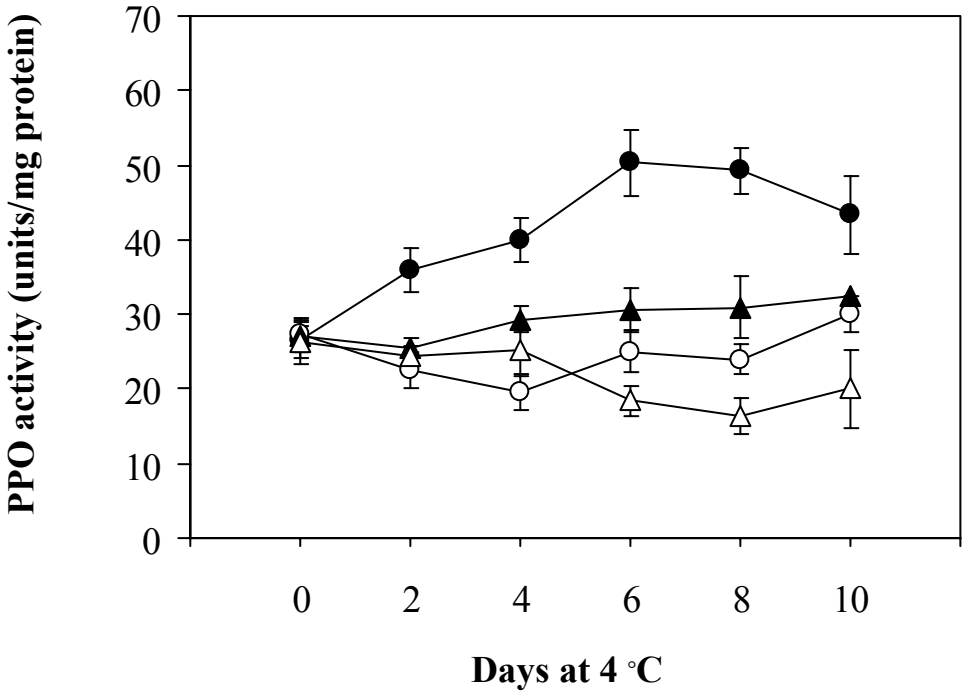

Figure 20 Changes in PPO activities of cv. Hom Thong banana peel with (○) and without (●) HW at 42ºC for 15 min and cv. Namwa banana peel with (△) and without (▲) HW stored at 4ºC. Data are means ± SE of three replications.

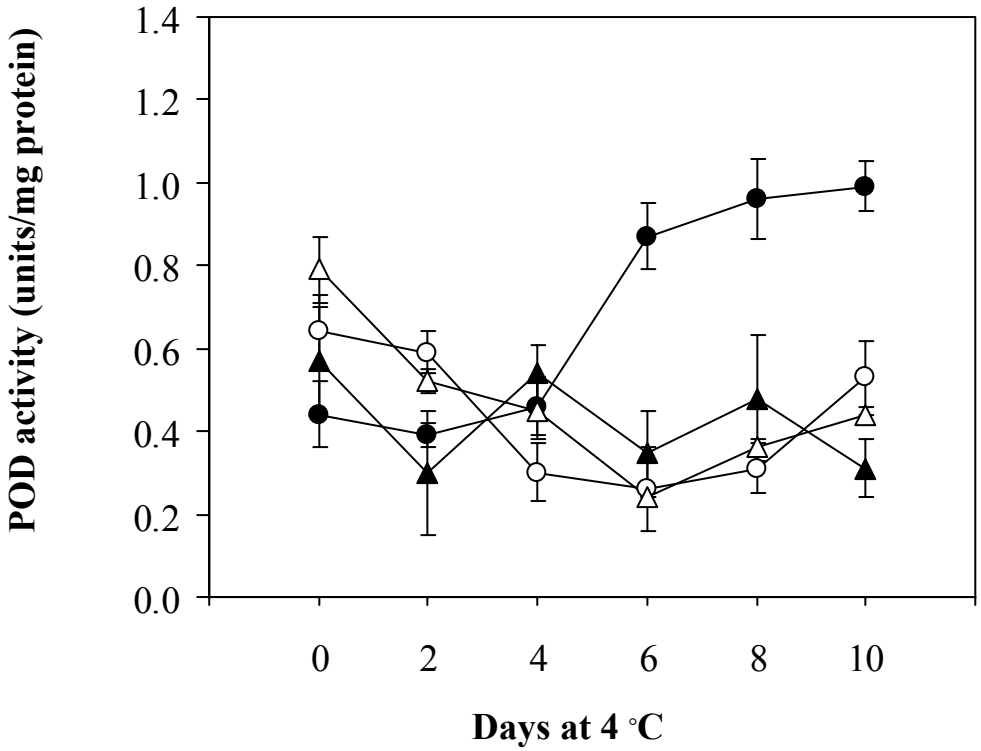

Figure 21 Changes in POD activities of cv. Hom Thong banana peel with (○) and without (●) HW at 42ºC for 15 min and cv. Namwa banana peel with (△) and without (▲) HW stored at 4ºC. Data are means ± SE of three replications.

Experiment 3: Effects of hot water treatments on expression of *PPO*, *LO*X and *Hsp70* genes in bananas cvs. Hom Thong and Namwa under low temperature storage

The sequences of *PPO*, *LOX*, *Hsp70* and 1*8S rRNA* genes in banana peel are given in Table 4. The bold letters with underline represent forward and reverse primers for product amplification.

Table 4 The size of amplified cDNA fragments and the sequences of PPO, LOX, Hsp70 and 18S rRNA genes

Gene	Product size (bp)	Sequence
PPO	243	**TCCACAACTCCTGGCTCTTC**TTCCCTTGGCACCGCTTCTAC CTCTACTTCCACGAGAGGATCCTCGGAAAGCTCATAGGCGA CGACACTTTCGCCCTCCCTTTCTGGAACTGGGACGCGCCCG GCGGCATGAAGCTGCCGTCGATCTACGCCGACCCTTCGTCC TCGCTCTATGACAAGTTTCGCGACGCCAAGCACCAGCCGCC GGTCCTCGTCGACCTCGA**CTACAACGGAACCGACCCTA**
LOX	246	**AGGAGTTTCCTCCGGTTAGC**AAGCTTGATCCGAAAGTATA TGGCGATCATACCAGCTCGATCAAAGCATCTCACATCGAGA AGAATCTCGAAGGCCTTACTGTGCAAAAGGCACTGAAGGA GAACAAGCTCTTCATTTTGGATCACCATGATGCCTTGATGC CGTACCTGAGGCGCATCAACTCTGGTTCCAACAAGATCTAC GCCAGTAGGACCCTGCTGCTGCT**CAAGGATGATGGCACTC TGA**
Hsp70	267	**TGAGGAGCTCAACATGGACCTGT**TCAGGAAGTGCATGGA TCCTGTCGAGAAGTGTTTGAGGGATGCCAAGATGGACAAG AGCAGTGTCGATGATGTGGTTCTTGTTGGTGGGTCTACCAG AATTCCAAAGGTTCAGCAACTGCTGCAGGACTTTTTCAATG GTAAGGAGCTATGCAAAAGTATTAATCCTGACGAGGCAGTT GCTTATGGTGCTGCTGTTCAGGCTGCAATTCTTAGCGGTGA GG**GCAATGAGAAGGTGCAGGACCT**
18S rRNA	216	**GTTGCAGTTAAAAAGCTCGT**AGTTGGACTTTGGGACGGGT CGGTCGGTCCGCCTCGCGGTGTGCACCGGTCGTCCCATCCC TTCTGTCGGCGATGCGTGCCTGGCCTTAACTGGCCGGGTCG TGCCTCCGGCGCTGTTACTTTGAAGAAATTAGAGTGCTCAA AGCAAGCCCACGCTCTGGATACATTAGCATGGG**ATAACAT CACAGGATTTCGG**

The sequences of *PPO, LOX* and *Hsp70* genes were translated to deduced amino acid using DNA to protein- translated program (FastPCR) and then both sequences of nucleotide and amino acid were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw). The 243 bp of partial cDNA encoding *PPO* gene of banana peel were identified. After alignment of the partially deduced amino acid sequences with other plants, *PPO* gene of banana shared 71-90 % homology with *Oryza sativa, Pyrus pyrifolia, Triticum aestivum and Ananas comosus*, respectively.

The partial cDNA encoding *LOX* gene of banana peel (246 bp) was identified, it shared 69-77% homology with *Corylus avellana, Solanum tuberosum, Pyrus pyrifolia* and *Prunus dulcis*, respectively. The partial of cDNA encoding *Hsp70* gene of banana peel was also identified, *Hsp70* gene of banana peel shared 90-92 % homology with *Zea mays, Pisum sativum, Oryza sativa and Solanum lycopersicum,* respectively. Highly conserved of *PPO*, *LOX* and *Hsp70* genes of banana peel are shown in Figure 22, 23 and 24

```
Banana          ----HNSWLFFPWHRFYLYFHERILGKLIGDDTFALPFWNWDAPGGMKLP 46
Ananas          EIQIHNSWLFFPWHRFYLYSNERILGKLIGDDTFALPFWNWDAPGGMQFP 248
Pyrus           ELQVHNSWLFFPFHRYYLYFFEKILGKLINDPTFAMPFWNWDSPAGMPLP 241
Triticum        ELQIHNCWLFFPWHRFYLYFHERILGKLIGDDTFALPFWNWDAPAGMKLP 234
Oryza           EIQIHSCWLFFPWHRMYLYFHERILGKLIGDETFALPFWNWDAPDGMSFP 238
                    *..*****:** ***  *:******.* ***:******:* ** :*

Banana          SIYADPSSSLYDKFRDAKHQPPVLVDLDYNGTDP---------------- 80
Ananas          SIYTDPSSSLYDKLRDAKHQPPTLIDLDYNGTDPTFSPEEQINHNLAVMY 298
Pyrus           AIYADPKSPLYDKFRSAKHQPPTLIDLDYNGTEDNVSKETTINANLKIMY 291
Triticum        VIYANRSSPLYDERRDPAHQPPVLVDLDYSGTDANIPRDQQIDENLKIMY 284
Oryza           AIYANRWSPLYDPRRNQAHLPPFPLDLDYSGTDTNIPKDQLIDQNLNIMY 288
                 **::  *.***  *.  * **  :****.**:
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Figure 22 Alignment of partial deduced amino acid sequence of banana peel *PPO* cDNA fragment with other plants, Ananas (AF261957), Pyrus (BAB64530), Triticum (ABK62804) and Oryza (ABG23059). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed, according to the table above. The dot (.) means that semi-conserved substitutions are observed.

```
Banana      ----------------------------------------EFPPVSKLDPKVYGDHTSS 19
Prunus      QLLRFPMPQVIEEDKSAWRTDEEFAREMLAGVNPVNISLLQEFPPASKLDPKVYGDQTSR 405
Pyrus       -----------------WRTDEEFAREMLAGVNPVNIARLQVFPPASKLDPKVYGDQNST 43
Corylus     GLLKYPMPQVIKEDKSAWRTDEEFGREMLAGVNPVNIRRLQEFPPASKLDPKVYGDQAST 416
Solanum     PFLKFPMPDVIKVDRSAWRTDEEFGREMLAGVNPVIIRRLQEFPPASKLDPEVYGNQTSS 416
                                                  ***.*****:***:: *

Banana      IKASHIEKNLEGLTVQKALKENKLFILDHHDALMPYLRRINSGSNKIYASRTLLLLKDDG 79
Prunus      ITEQDIGNKLDGLTVHEALKQNKLFILDHHDALMPYLRRINSTSNKIYASRTVLFLKSDG 465
Pyrus       ITEEHIKNNLDGLTVDEALKKNKLFILDHHDSLMPYLRRINSTSNRIYGSRTLLFLQNDG 103
Corylus     ITKEHIENNIDGLSIDEAINKKKLFILDHHDAIMPYLRRINSTSTKTYASRTILFLKNDG 476
Solanum     IKREHIEKNMDGLTVDEAIECNRLFILDHHDALLPYLRRINTTKTKTYATRTLLYLQDNG 476
            *. ..* :::::**::.:*:: ::********:::********: ..: *.:**:* *:.:*

Banana      TL------------------------------------------------------------ 81
Prunus      TLKPLVIELSLPHPDGDQFGRISKVYTPAEEGVEGSIWQLAKAYVAVNDSGYHQLISHWL 525
Pyrus       TLKPLVIELSLPHPDGDQFGCISNVYTPAEQGVEGSIWQLAKAYVAVNDSGVHQLISHWL 163
Corylus     TLKPLVIELSLPHPEGDQFGAISKVFTPAEEGVESSIWQLAKAYVAVNDSGYHQLISHWL 536
Solanum     TLRPLAIELSLPHPQGDKHGATSLVFTPADEGVEGTVWQLAKAYAAVNDSGYHQLISHWL 536
             **
```

Figure 23 Alignment of partial deduced amino acid sequence of banana peel *LOX* cDNA fragment with other plants, Prunus (CAB94852), Pyrus (ABN09736), Corylus (CAD 10740) and Solanum (AAD09202). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed, according to the table above. The dot (.) means that semi-conserved substitutions are observed.

```
Banana          ------PFEELNMDLFRKCMDPVEKCLRDAKMDKSSVDDVVLVGGSTRIP 44
Zea             STITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTVHDVVLVGGSTRIP 116
Oryza           STITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSSVHDVVLVGGSTRIP 349
Solanum         STITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTVHDVVLVGGSTRIP 350
Pisum           SPITRARFEELNMDLFRKCMEPVEKCLRDAKMDKKSIHDVVLVGGSTRIP 349
                       *************:*************.:::.************

Banana          KVQQLLQDFFNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKVQDLRAN 94
Zea             RVQQLLQDFFNGKELCKNINPDEAVAYGAAVQAAILSGEGNEKVQDLLLL 166
Oryza           RVQQLLQDFFNGKELCKNINPDEAVAYGAAVQAAILSGEGNEKVQDLLLL 399
Solanum         KVQQLLQDFFNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKVQDLLLL 400
Pisum           KVQQLLQDFFNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKVQDLLLL 399
                :****************.****************************
```

Figure 24 Alignment of partial deduced amino acid sequence of banana peel *Hsp70* cDNA fragment with other plants, Zea (CAC16168), Oryza (CAA47948), Solanum (CAA37971) and Pisum (CAA83548). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means the conserved substitutions have been observed, according to the table above. The dot (.) means that semi-conserved substitutions are observed.

3.1 Semi-quantitative RT-PCR

Expression of the gene encoding *PPO* in cv. Hom Thong, was observed from day 2 of storage at 4°C, increased in abundance until day 8 and then showed a large decreased abundance on day 10 (Figure 25). The *LOX* gene showed high expression on day 0, with a decrease in abundance subsequent sampling point. After the HW treatment and storage at 4°C (Figure 26), the abundance of the *Hsp70* mRNA progressively increased was found more in cv. Hom Thong than cv. Namwa. It was very high from day 2 and remained high until day 10 (Figure 27). Except on day 10, no clear band of *PPO* mRNA was found after HW treatment, and a decrease in the abundance of *LOX* mRNA, compared with bananas without HW treatment (Figure 25). *PPO* mRNA in cv. Namwa stored at 4°C was found highest expression on day 6 and then decreased thereafter, whereas bananas with HW treatment the expression of *PPO* gene showed no clear changes in the mRNA abundance of this gene (Figure 28). Accumulation of *LOX* mRNA in cv. Namwa without HW treatment maintained constant until day 4 then increased to a maximum on day 10. The expression of *LOX* gene in cv. Namwa was suppressed by HW treatment on day 2 and day 6 (Figure 29). *Hsp70* mRNA in cv. Namwa without HW treatment was detectable on day 2 and barely detectable on day 4 and day 6 then increased expression again until the end of the experiments, while *Hsp70* mRNA in cv. Namwa after HW treatment was found highest expression on day 2 then sharply decreased thereafter (Figure 30).

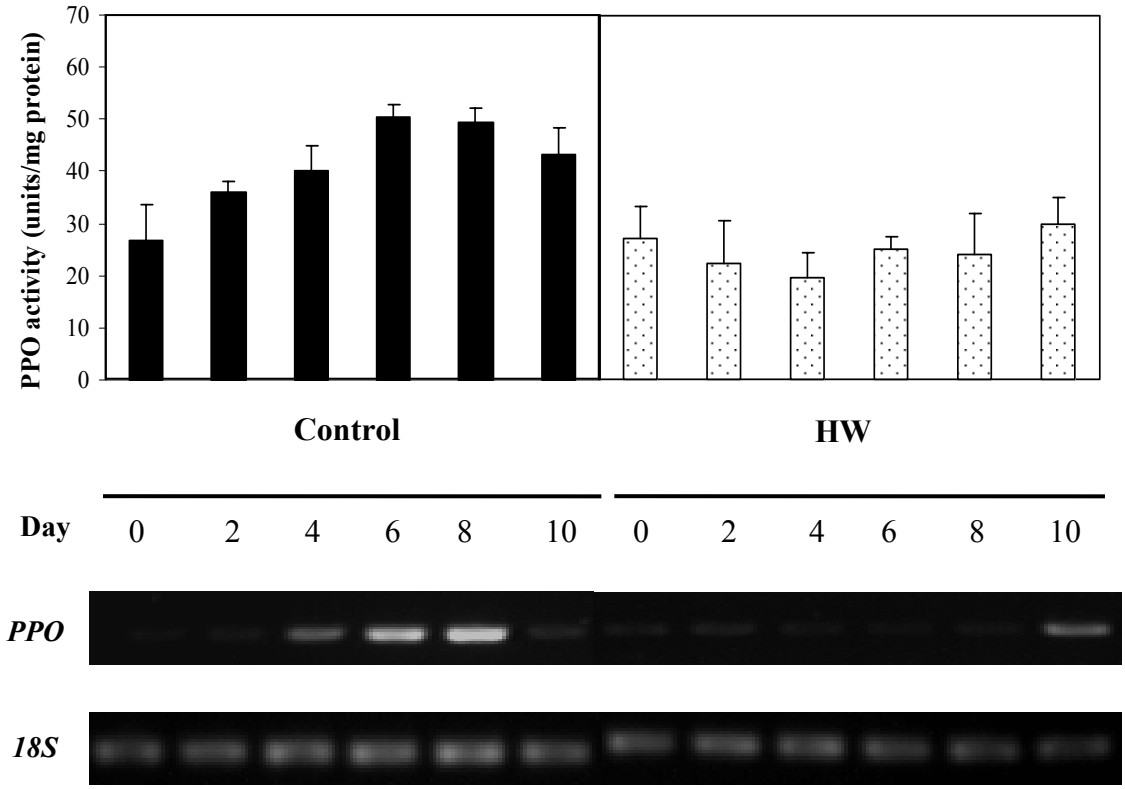

Figure 25 Semi-quantitative RT-PCR of *PPO* expression in the peel of cv. Hom Thong stored at 4°C with and without HW at 42°C for 15 min.

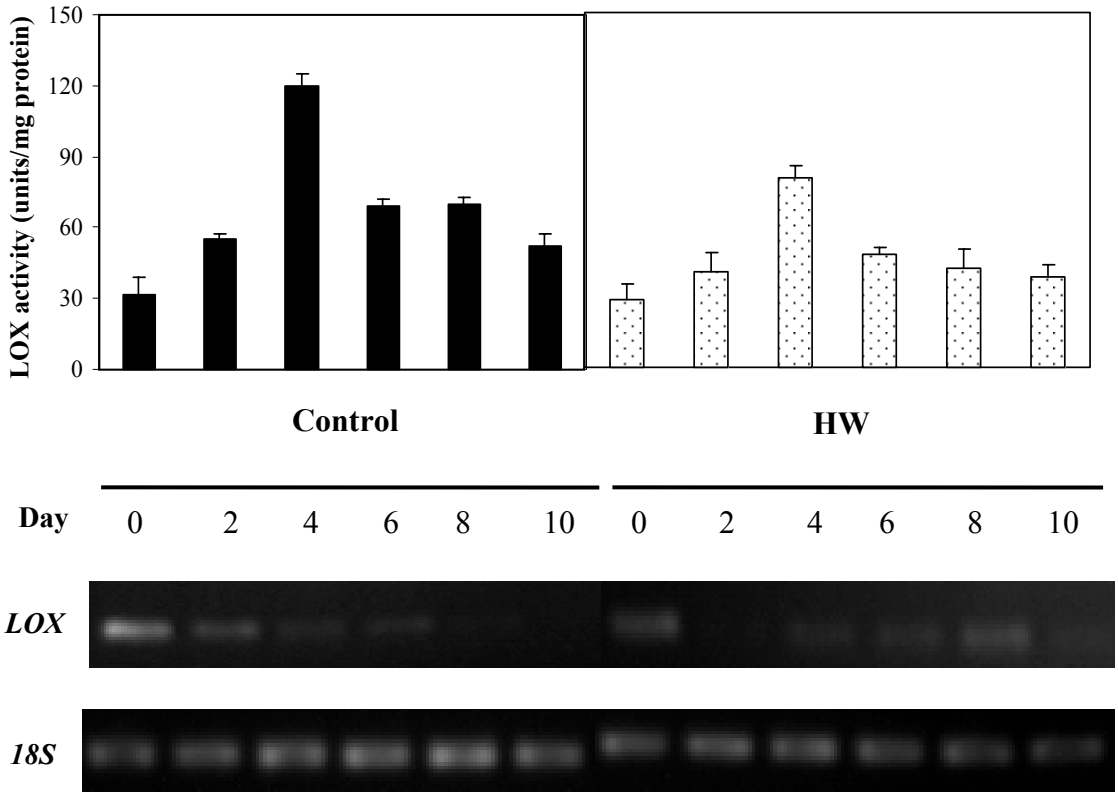

Figure 26 Semi-quantitative RT-PCR of *LOX* expression in the peel of cv. Hom Thong stored at 4°C with and without HW at 42°C for 15 min.

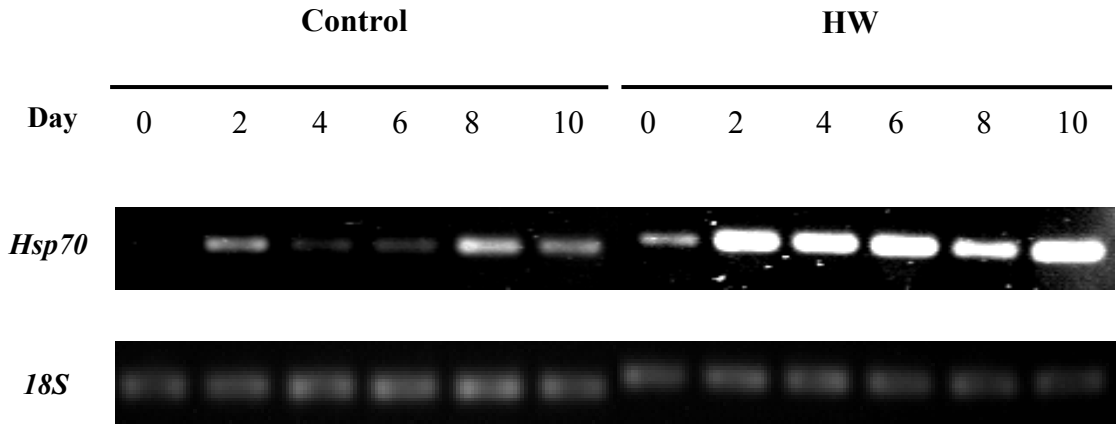

Figure 27 Semi-quantitative RT-PCR of *Hsp70* expression in the peel of cv. Hom Thong stored at 4°C with and without HW at 42°C for 15 min.

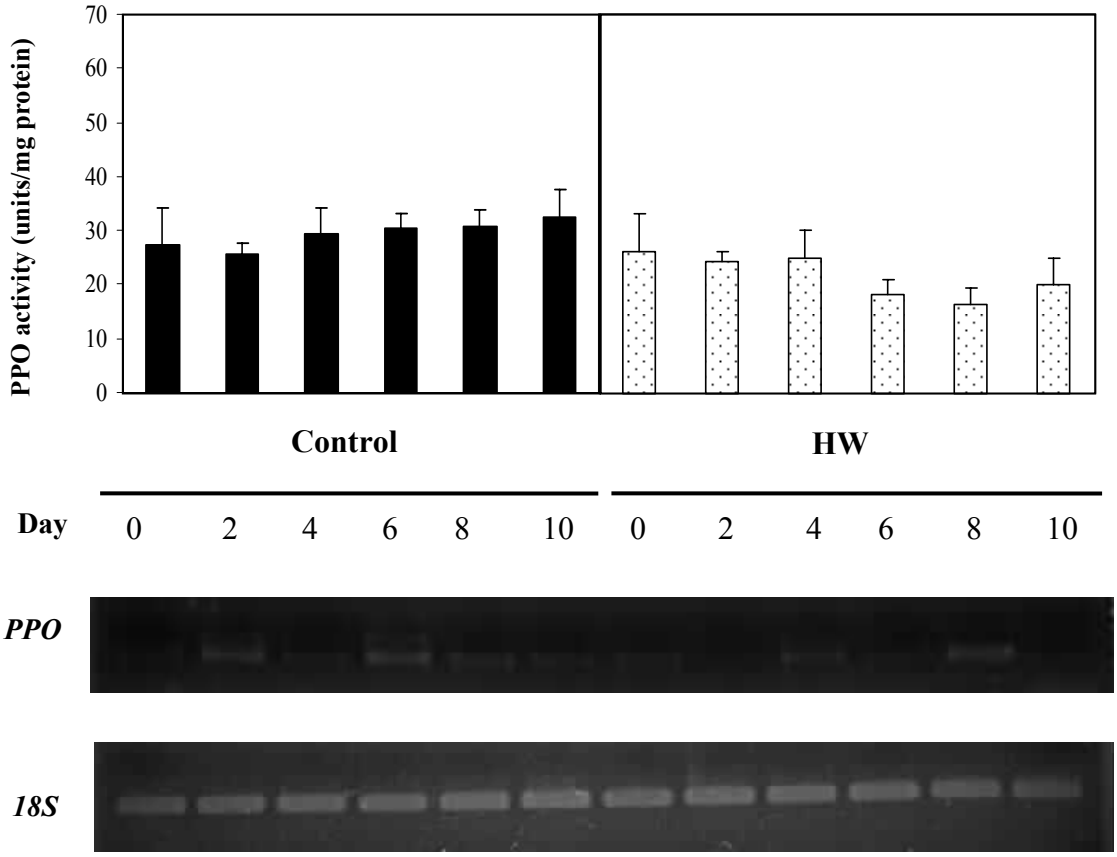

Figure 28 Semi-quantitative RT-PCR of *PPO* expression in the peel of cv. Namwa stored at 4°C with and without HW at 42°C for 15 min.

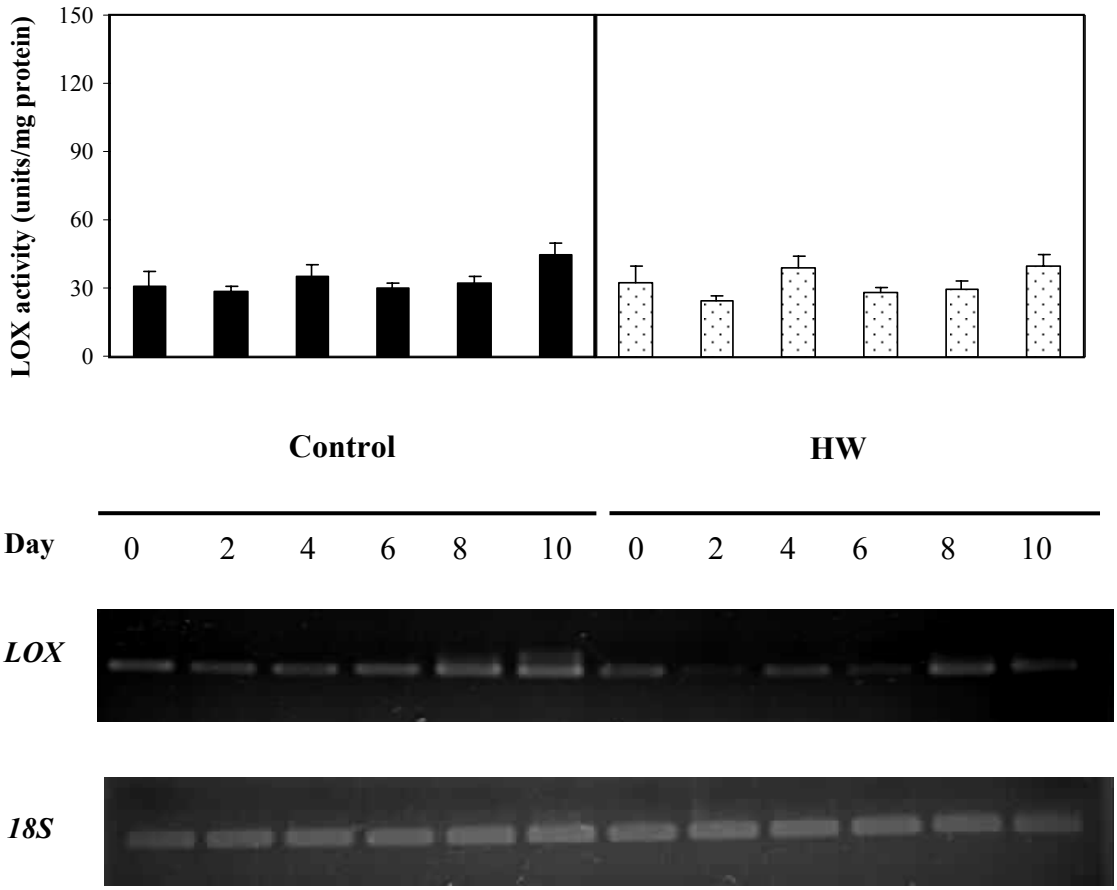

Figure 29 Semi-quantitative RT-PCR of *LOX* expression in the peel of cv. Namwa stored at 4°C with and without HW at 42°C for 15 min.

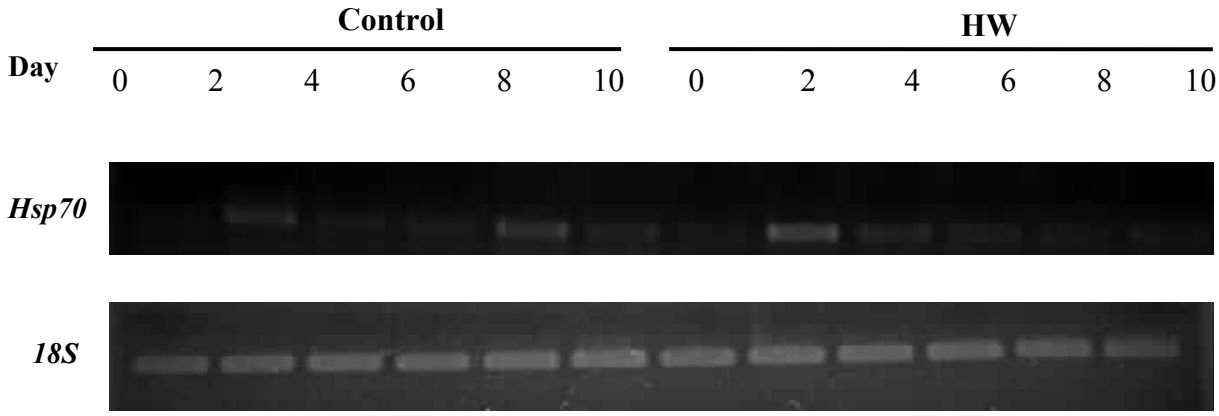

Figure 30 Semi-quantitative RT-PCR of *Hsp70* expression in the peel of cv. Namwa stored at 4°C with and HW at 42°C for 15 min.

3.2 Real-time PCR

3.2.1 Relative expression of the *PPO* gene

Relative expression of the *PPO* gene in cv. Hom Thong without HW treatment stored at 4°C increased continuously to a maximum on day 8 then drastically decreased on day 10, whereas in cv. Hom Thong with HW treatment was found the highest level of expression at the end of storage (Figure 31A). In cv. Namwa with HW treatment relative expression increased sharply on day 6 and then decreased thereafter, while in cv. Namwa without HW was stable at low level until day 6 then increased to a maximum on day 8 and decreased at low level again at the end of storage (Figure 31B).

3.2.2 Relative expression of the *LOX* gene

Relative expression of the *LOX* gene in cv. Hom Thong without HW treatment stored at 4°C was already found the highest level on day 0 after that it decreased throughout the storage period. Expression in cv. Hom Thong with HW also found the highest level on day 0 then decreased thereafter until day 6, it slightly increased on day 8 and decreased again at the end of storage (Figure 32A). Relative expression of

the *LOX* gene in cv. Namwa without HW treatment stored at 4°C remained unchanged during day 0 to day 6 and then increased continuously to a maximum at the end of storage, whereas relative expression in cv. Namwa with HW treatment at 4°C fluctuated (Figure 32B).

3.2.3 Relative expression of the *Hsp70* gene

Relative expression of the *Hsp70* gene in cv. Hom Thong without HW treatment stored at 4°C was observed on day 2 and slightly decreased on day 4 to day 6 then increased to a maximum on day 8 and decreased again at the end of storage. While relative expression of the *Hsp70* in cv. Hom Thong with HW was found the maximum level on day 2 and the fluctuated throughout the storage time (Figure 33A). Relative expression of the *Hsp70* gene in cv. Namwa without HW treatment remained the low level during day 2 to day 6 then increased to a maximum level on day 8 and decreased thereafter, while in cv. Namwa with HW treatment had the highest level on day 2 and then decreased throughout the storage time (Figure 33B).

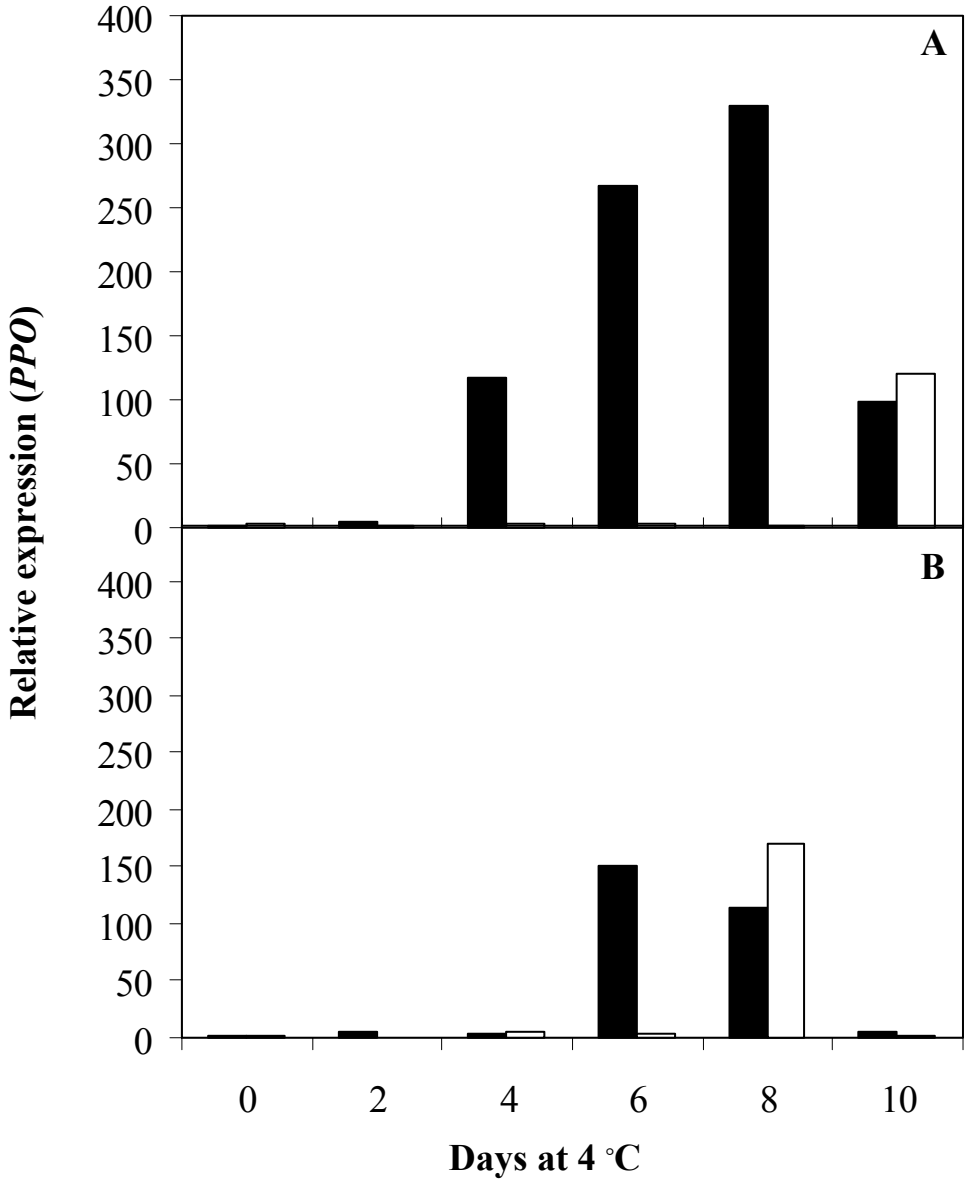

Figure 31 Relative expression of PPO gene in cv. Hom Thong banana peel (A) and cv. Namwa banana peel (B) stored at 4°C with (□) and without (■) HW for 10 days.

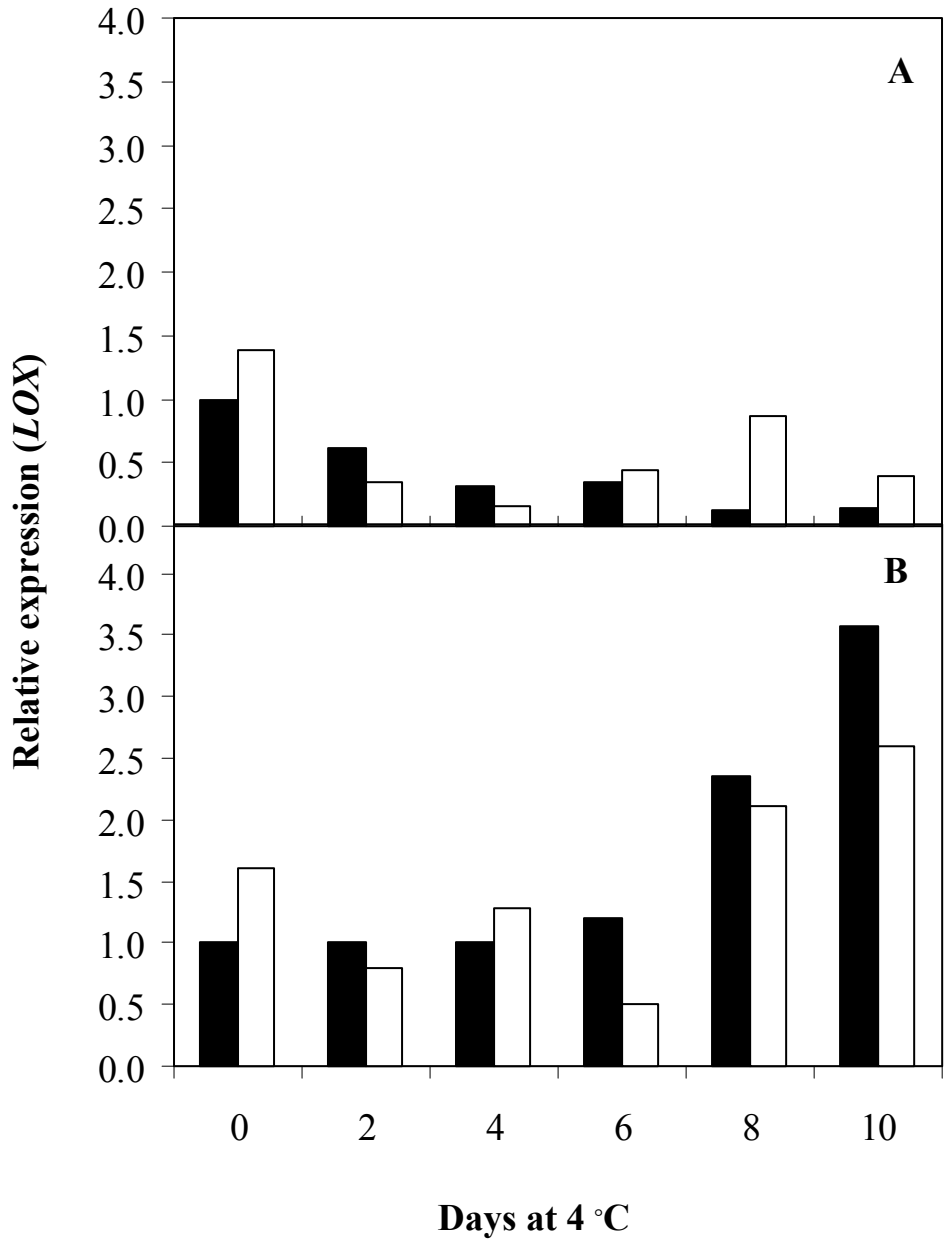

Figure32 Relative expression of *LOX* gene in cv. Hom Thong banana peel (A) and cv. Namwa banana peel (B) stored at 4°C with (□) and without HW for 10 days.

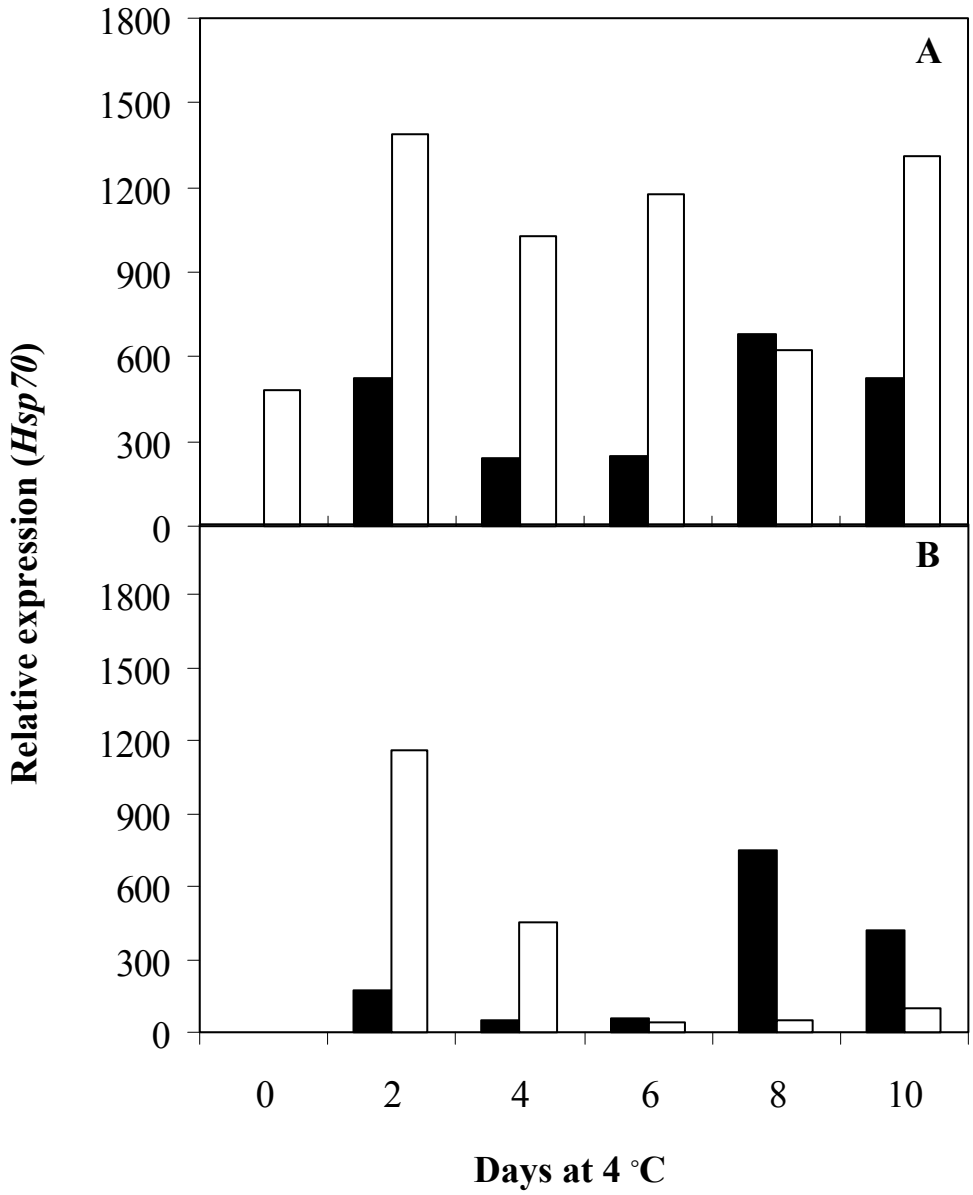

Figure 33 Relative expression of *Hsp70* gene in cv. Hom Thong banana peel (A) and cv. Namwa banana peel (B) stored at 4 °C with (□) and without (■) HW for 10 days.

DICCUSSION

1. Effect of low temperature on physiological and biochemical changes of two banana cultivars in relation to chilling injury

Chilling injury of banana fruit is a physiological disorder and can easily occur at low temperature storage depending on the particular species and cultivars. In both banana cultivars, Namwa and Hom Thong CI occurred more rapidly at 4ºC than that at 12ºC. The results showed that banana cv. Hom Thong (*Musa acuminata,* AAA Group) was more sensitive to chilling-induced peel blackening than that in banana cv. Namwa (*Musa* x *paradisiaca*, ABB Group) (Figure 5). Although banana cv. Namwa is in the triploid group as banana cv. Hom Thong but they are different in the genome type (Valmayor, *et al*., 1990). This result suggested that banana with B genome type may less sensitive to CI than banana with A genome type. Therefore, chilling sensitive character may be correlated with the number and type of banana genome. At the present, there is no evidence to support this assumption. However, with regard to the cross-section tissues of banana fruits after storage at 4ºC for 2 days, it was found that the latex vessels in cv. Hom Thong appeared closer to each other whereas in cv. Namwa were scatter from one to another. This might be related to CI susceptibility (data not shown). Regardless to genome type, bananas stored at 12ºC showed a brighter green color than those stored at 4ºC according to their L values and it was also found that a decrease in L values at 4ºC was more in cv. Hom Thong than in cv. Namwa (Figure 7A and 7B). This indicated that CI symptoms development of banana was negatively correlated with L value, resulting in peel blackening of bananas.

The response of plant tissues to CI has been frequently separated into primary and secondary responses. Two distinct possibilities are suggested as the primary event such as physical change in membrane lipids (Paull, 1990). Previous studies have suggested that cell membranes are the first structures to be damaged when plants suffer from chilling injury (Lyons, 1973). As the primary event leading to visible CI might be due to membrane damage, it possibly relates to fatty acid composition, and phospholipid degradation. We monitored the relationship between visible damage and

parameters related to membrane composition and degradation. According to the theory, a higher ratio of unsaturated to saturated fatty acids (UFA/SFA) provides higher tolerance to low temperature (Lyons *et al.* 1964). Previous studies have shown that low temperature has differential effects on the lipid composition of membranes in chilling-sensitive and chilling-tolerant plants. For example, the proportion of unsaturated fatty acids tends to increase in the thylakoid membrane, mitochondrial membrane, and plasma membrane during low temperature stress (Larkindale and Huang, 2004). These relationships seem confirmed the present study. The ratios of UFA/SFA in the peel of both cvs. Hom Thong and Namwa did not change much during storage at 12ºC for 8 days, suggested that phase transition of fatty acids in membranes of both cvs. Hom Thong and Namwa did not occur yet and this is why both banana cultivars did not show severely CI on day 8 at storage of 12ºC. However, the ratios of UFA/SFA in the peel of cv. Hom Thong stored at 4ºC for 8 days were lower than that of cv. Namwa, suggested that lipid membranes of cv. Hom Thong banana peel are prone to change under chilling temperature much easier than that of cv. Namwa banana peel resulting in more sensitive to CI of cv. Hom Thong than cv. Namwa. These data strongly suggest that an increase in the degree of unsaturated fatty acids of membrane lipids correlates positively with chilling tolerance of the banana peel. The results of this study also agree with the study on the relationship between the increase of fatty acid desaturation and the severity of CI in tomato (Whitaker, 1994). Similarly, Dalmannsdottir *et al*. (2001) have reported that plants with higher percentage of unsaturated fatty acids in cell membranes have higher survival potential at low temperature.

In plants, peroxidation of unsaturated fatty acids and degradation of lipid membranes might be among the initial biochemical effects of CI induced by low temperatures. These processes have been attributed to LOX activity (Alonso *et al*., 1997). The present study found an early increase in LOX activity in the peel of cv. Hom Thong, which is sensitive to CI stored at 4ºC accompanied peel blackening from day 2. However, the increase to a maximum level of LOX activity in cv. Namwa by day 10 might be too late to account for the peel blackening from day 4 (Figure 11B). Although the slight decrease in LOX activity of cv. Hom Thong during the last period stored at 4ºC may resulted from the increased of peroxidation reaction on membrane

lipid, which could decrease LOX substrates. While both banana cultivars stored at 12ºC, the activity of LOX remained constant throughout the storage period (Figure 11A). This may indicate that LOX activity plays an important role in membrane damage of chilling sensitive banana (Berger *et al*., 2001).

In regard to membrane leakage, electrolyte leakage and MDA content (TBA-reactive compounds) have been often reported to increase in chilling sensitive plants and used as indirect indicators of membrane damage to determine the extent of CI. Therefore, strongly increasing electrolyte leakage in the peel of cv. Hom Thong subjected to CI may also result from damage of membrane components (Figure 8A and 8B). A higher susceptibility of cv. Hom Thong to CI evidenced by the strong leakage increased during chilling was related to MDA content, indicated that the occurrence of lipid degradation and enhanced membrane permeability, may cause the promotion of an enzyme and substrate contact induced by CI. Moreover, MDA is a major product of membrane fatty acid oxidation and can act as a suitable index of membrane integrity (Dhindsa, 1991). These results confirmed again that CI is related to lipid peroxidation and the results were similar to the report of Campos *et al*. (2003) who found electrolyte leakage and MDA content increased in cold sensitivity of coffee leaves. In addition, earlier studies reported that tomatoes analyzed immediately after chilling showed higher electrolyte leakage in chilling-sensitive lines than in chilling-torelance lines (King and Ludford, 1983). Many scientists have also presented data indicating that increased membrane permeability and increased rates of electrolyte leakage are associated with CI of sensitive tissues (Murata and Tatsumi, 1979; Paull, 1981; Kuo and Parkin, 1989; Saltveit, 2000a). Furthermore, cv. Namwa presented a chilling-resistance and a good ability to maintain membrane integrity under low temperature, evidenced by the stability of lipid contents and slight permeability changes.

Saltveit and Morris (1990) reported that CI symptoms in many fruits and vegetables are often associated with metabolism of phenolic compound. Banana peel reportedly contains free phenols such as dopamine (Udenfriend *et al*., 1959; Riggin *et al*., 1976), tyramine (Udenfriend et al., 1959; Wallrauch and Pflaum 2005), and norepinephrine (Riggin *et al*., 1976). Neither L-dopa (L-3,4-dihydroxyphenylalanine)

nor epinephrine were present in banana fruits (Udenfriend *et al*., 1959; Riggin *et al*., 1976). Phenolic compounds act as substrates for a number of oxidoreductases, namely PPO and POD. It is known that, at the subcellular level, phenolics are present mainly in the vacuole, while PPO and POD are synthesized in the cytoplasm and may be also deposited in cell walls (Vamos-vigyazo, 1981; Walker and Ferrar, 1998). There are several evidences suggesting that breakdown of cellular compartments is apparently required the enzyme to react with the substrate. If low temperature induced membrane damage of cell organelles, such as vacuoles, the vacuole phenolics might possibly contact with PPO, thus causing the browning reaction in plant cells.

In present study, visible symptoms of CI in the banana peel, mainly browning occurred at 4ºC, especially in cv. Hom Thong. The results showed no significant difference in the level of total free phenolics, either between the cultivars or between the two storage temperatures (Figure 10A and 10B). Similarly, 'Delicious and 'Ralls' apples, held for 4-5 months in cold storage had no changes in the concentrations of phenolics (Ju *et al*., 1996). Other reports have also shown the total phenolics in apples remained relatively stable during cold storage (Burda *et al*., 1990). The results suggested that free phenolics content is not a limiting factor differing in susceptibility to CI of both banana cultivars. Although it was found no effect of low temperature on total free phenolics. One of the reasons may be that the quinones formed by oxidation can react with ascorbic acid resulting in regeneration of phenols (Rouet-Mayer *et al*., 1990). However, it appears to be no discussion of how phenolics recovery influenced by low temperature. On the other hand, it is possible that the analysis of total free phenolic compounds determined as absorbance of methanol extraction at 280 nm, represents a non-specific analysis that it is not able to detect the products of oxidation catalysed by PPO and POD.

In plant, PPO is encoded by nuclear genes and predominantly located in the chloroplast thylakoid membranes while its substrates are located (phenolic compounds) in the vacuole. Therefore, PPO mediated browning reactions occured after a loss of compartmentation (Hind *et al*., 1995). Various isoforms of PPO may have different substrate specificities. The main PPO substrate depends on the commodity. For example, flavan-3-olmonomers and dimmers are the main PPO

substrates in litchi (Zhang *et al*., 2000), 3,4-dihydroxyphenylalanine (DOPA) is a substrate for PPO in potato (Chen *et al*., 1991), *p*-coumaryl and caffeoyl-tartaric acid are substrate for PPO in grape, and chlorogenic acid is a substrate for PPO in peach and nectarine (Cheng and Crisosto, 1995). PPO isolated from the banana peel had been showed very high activity when using dopamine or tyramine as substrate (Yang *et al*., 2001, 2004). Therefore, in the experiment used dopamine as a substrate and assume that substrate also provided a suitable substrate to measure PPO activity in banana peel. Free phenolic, when oxidized by the enzyme PPO are responsible for the rapid browning of banana tissue (Palmer, 1971). Similarly, we found that, the activity of PPO in cv. Hom Thong stored at 4ºC dramatically increased higher than that in cv. Namwa while their PPO activity of both banana cultivars stored at 12ºC did not change significantly during storage period (Figure 12A and 12B), suggested that CI induced peel blackening is causally related to PPO activity. The result was similar to the report of Nguyen *et al*. (2003) showed that peel blackening in cv. Sucrier (*Musa* AA Group) and cv. Hom Thong stored at 6ºC was associated with a small increase of *in vitro* PPO activity.

POD can oxidize phenols to quinones, then condense quinones to brown polymers in the presence of H_2O_2, which may also contribute to browning (Robinson and Dry, 1992). Increased POD activity has been observed upon exposure to chilling injury (Campa, 1991). However, in contrast to PPO, the results showed in the two banana cultivars studied, were the changes of POD activity in the peel stored at 4ºC steadily increased similar to that stored at 12ºC. POD activity showed no significant change after chilling injury even though cv. Hom Thong had higher POD activity than cv. Namwa (Figure 13A and 13B). These results suggested that POD may not be associated with CI development in banana and the level of POD cannot be used as an indicator of browning potential. Similar results have been reported in avocado fruit stored at chilling temperature (Zauberman *et al*., 1988). Moreover, the present study agreed with previous report by Zhou *et al*. (2003), who found that POD is not a key-browning enzyme causing blackheart development in pineapple fruit after chilling.

2. Effect of hot water treatments (HW) on reducing CI of banana during low temperature storage

Bananas exhibit many physiological and biological changes when exposed to low temperature as shown in Experiment1. Recent studies have demonstrated that CI susceptibility of banana is related to compartmentation and loss of membrane function in the cell by lipid oxidation enzyme with consequent phenol oxidation. Several methods of heat treatments including hot water, forced hot air and vapour can induce tolerance to low temperature, thus reducing CI during storage and increasing shelf life of many commodities (Lurie, 1997; Ferguson *et al*., 2000; Fallik, 2004). In recent study observed that, hot water treatment had more efficiency on reducing CI in bananas than others methods of heat treatments. Heated fruit did not show visible peel damage related to CI and the taste of the flesh was not affected by hot water treatment (data not showed). While hot air treatment was not suitable and particularly effective on banana to reduce CI. Hot air showed less heat penetration than hot water and caused severity of heat damage to bananas (Lurie, 1998).

CI evaluation of bananas in the experiments observed by peel blackening, electrolyte leakage and fruit peel color (L values). However, there symptoms were significantly reduced in both cultivars with HW treatment at 42ºC for 15 min. Thus, at the end of the experimentation, the scores of CI evaluation in cvs. Hom Thong and Namwa without HW treatment (control) were 5.0 and 4.0 respectively, while in cvs. Hom Thong and Namwa with HW treatment were 3.9 and 3.2, respectively (Figure 15A and 15B). In the experiment, electrolyte leakage was significantly higher in cv. Hom Thong without HW treatment than in other treatments over storage period (Figure 17). Similarly, HW treatment of tomato discs (Saltveit, 2005) and strawberry fruit (Vicente *et al*., 2006) showed reductions in electrolyte leakage during short or long storage periods. In addition, after storage avocado fruits at 0°C, less ion leakage was observed in skin tissue taken from exposed sides of fruit in HW treatment, concomitantly with less postharvest chilling injury (Woolf *et al*., 1995). An effect of high preharvest fruit temperatures on membrane properties has also been suggested by Paull and Chen (2000).

So far as increased membrane leakage is a widespread of chilling injury symptom and heat treatments can reduce this phenomenon when applied before and after the chilling treatment (Saltveit, 2000b). These indicated a high correlation between peel blackening and electrolyte leakage. HW treatment resulted in membranes became more resistant to low temperature damage. In the present study with two banana cultivars, HW treatment prior to storage at low temperature maintained membrane integrity (Figure 17) and protected bananas CI against membrane damage (Figure 15).

The increased peel blackening in both cultivars was also observed by a reduction of L values, and was prevented by HW treatment (Figure 16). The results of present study confirm that hot water treatments can reduce the sensitivity of banana to low temperature, especially in cv. Hom Thong. This suggested that HW could induce acclimation of banana to low temperature and in turn reduce chilling injury. This finding was similar to other fruits such as in avocado (Woolf *et al*., 1995), tomato (McDonald *et al*, 2000), orange and citrus fruit (Porat *et al*., 2004), grapefruit (Dafna *et al*., 2004), cucumber (Saltveit *et al*., 2004) and pomegranate (Mirdehghan *et al*., 2007).

In the banana peel, six fatty acids were identified and quantified, three saturated (C16:0, C18:0 and C22:0) and unsaturated (C18:1 *trans*, C18:2 *cis* and C18:3) (Table 3A). The concentrations of unsaturated fatty acid in both cultivars with HW treatment remained significantly higher than in the control over storage period (Table 3B). The ratio of UFA/SFA decreased in control without HW treatment from initial value of 0.67 to 0.53 in cv. Hom Thong and from 1.08 to 0.96 in cv. Namwa at the end of sampling dates, while the ratio of UFA/SFA increased and higher ratios were found in both cultivars with HW treatment. This increase in ratio of UFA/SFA in both cvs. Hom Thong and Namwa with HW treatment was similar to the report found in pomegranate fruits (Mirdehghan *et al*., 2007). It is possible that, HW treatment may have ability to maintain unsaturated fatty acids during low temperature storage resulting in reduction of CI in bananas.

Both MDA content and the activity of LOX have been used as indicators of membrane degradation and often used as a measure of CI. The MDA content in cv.

Hom Thong increased within 2 days of storage at 4 ºC (Figure 18A). This increase was largely prevented by the HW treatment. However, HW treatment was less effective in cv. Namwa compared with the unheated control fruit. Only in cv. Hom Thong the effect of HW treatment was able to lower LOX activities (Figure 18B). By reducing MDA content and LOX activity, HW treatment probably, prevented the disruption of the cell membranes and maintained the cell compartments intact. Therefore, the PPO enzyme could not interact with its phenolic substrates, and subsequently resulting in less peel blackening in banana. No such correlation was found in cv. Namwa, suggested that LOX activity may not change with CI development in tolerance cultivar. A higher LOX activity is also related to production of aroma volatiles (Boyes *et al*., 1992). In the present study, no change in taste was found because of the cold storage or HW treatments, when no visible peel blackening was present. Nonetheless, cv. Hom Thong had higher LOX activity and more aroma than that of cv. Namwa.

Visible blackening of the banana peel suggests accumulation of polyphenols, oxidized by enzyme PPO, using free phenols as substrate. Previous study indicated that senescent spotting of cv. Sucrier (*Musa acuminata*, AA Group) peel was well correlated with total free phenolics (Choehom *et al*., 2004). Whereas there was no correlation found between total free phenolics and the degree of peel blackening during storage at low temperature in cvs. Hom Thong and Namwa with and without HW treatment (Figure 19). It is unclear if accumulation of free phenolics is a cause or a result of the reaction leading to the peel blackening in bananas. In the present study, it was found a lower activity of PPO observed in both cultivars, after HW treatment and was well correlated with reduction of visible peel blackening (Figure 20). This suggested that the activity of this enzyme might, under these conditions, limit the rate of blackening. This observation is in agreement with the report of Loaiza-Velarde *et al*. (1997) who found that the reduction of PPO activity has been observed in lettuce after heat treatment.

Results from the Experiment 1 showed that the POD activity was not associated with CI in banana. In the case of Experiment 2, after HW treatment of both cultivars, the results showed POD activity remained stable and no difference in POD

activity was found in all treatments except in cv. Hom Thong control fruits (Figure 21). Although, peroxidases are widely distributed in the plant kingdom and have been shown to participate in different physiological processes like lignification and wound healing (Wakamatsu and Takahama, 1993; Bunsiri *et al*., 2003; Dangcham, 2007). But this enzyme does not appear to participate in CI of bananas studies here under low temperature storage.

3. Effects of hot water treatments on expression of *PPO*, *LO*X and *Hsp70* genes in cv. Hom Thong and cv. Namwa banana under low temperature storage

Several sensitivity plants to low temperature depend on the modulation (up-regulation or down-regulation) of specific sets of genes are associated with the development of chilling tolerance. The results from semi-quantitative RT-PCR and Real time PCR showed that gene expression data of an increase in the mRNA abundance of a PPO gene from day 4 to day 8, in the unheated peel of cv. Hom Thong, while decreased in heat treated banana stored at 4°C (Figure 25).
The result showed similar trend of PPO mRNA induced by chilling in pineapple fruits (Stewart *et al*., 2001). In cv. Hom Thong the gene expression data indicated some correlations with peel blackening. The rise of PPO activity in cv. Hom Thong was associated with increased expression of the mRNA abundance of PPO gene, indicating that part of the increase in enzyme activity is regulated at the transcriptional level. A gene encoding *LOX* of cv. Hom Thong showed high abundance on day 0 and lower abundance thereafter. This seemed correlated well with a high enzyme activity on day 4 and a sharp decrease from day 6 (Figure 25), indicating that the enzyme activity was regulated, at least in part, by gene expression.

In the study, HW treatment induced high mRNA abundance of a *Hsp70* gene, which paralleled to the decrease in peel blackening suggesting that HW treatment induced synthesis of Hsps may be involved in protection of the banana peel against cold-induced blackening. Usually, under ambient temperature, most Hsps cannot be detected but are up-regulated by elevated temperatures and their mRNAs are usually most abundant during the first few hours of heat shock and then decline (Vierling, 1991). This finding confirmed that Hsp transcripts are up-regulated as a result of postharvest heat treatments of fruit such as papaya, tomatoes and avocado (Paull and

Chen, 1990; Lurie and Klein, 1991; Woolf *et al*., 1995). It is possible that these Hsp become associated with membranes as a result of heat-induce changes in cellular architecture and aid in maintaining normal membrane-associated processes during HW treatment (Ho and Sachs, 1989) resulting in reduction of CI in bananas caused by membrane permeability. Moreover, HW treatment also reduced the expression of both the genes encoding *PPO* and *LOX*. This was also in accordance with enzyme activities after HW treatment. Inhibition of enzyme synthesis by HW treatment might be the result from a redirection of protein synthesis away from CI-induced proteins (e.g., PPO or LOX) to the synthesis of heat shock proteins (Saltveit, 2000b). Remarkably, very little effect of HW treatment was found in the expression of these genes in cv. Namwa. This might indicate lack of a differential expression of gene encoding a PPO gene and might reflect the small changes found in PPO enzyme activity. This also indicated that HW treatment appears to be critical in suppressing normal protein synthesis while inducing the synthesis of Hsps. For example, tobacco cell suspension cultures heat shocked at 38°C produced Hsps without inhibition of the synthesis of normal proteins while at a heat shock of 42°C normal proteins were produced at a reduced rate (Kanabus *et al*., 1984). The correlation between the synthesis of Hsp and CI response led to the hypothesize that Hsp protect cells against the detrimental effect of CI stress could be through the interruption of altered normal metabolic processes or through inhibiting the synthesis of enzyme related to deleterious pathways of CI. The mechanisms by which Hsps are involved in cell protection are not fully understood. Strong evidence support that Hsps function as molecular chaperones that bind to partially folded or denatured substrate proteins and thereby prevent irreversible aggregation or promote correct substrate folding (Sun *et al*., 2002). More unexpected is the absence, in cv. Namwa, of an increase in *Hsp70* mRNA abundance after HW treatment (Figure 26). Whether or not persistence of *Hsp70* at low temperature is responsible for some of the chilling tolerance induced by heat treatments is not yet unclear.

CONCLUSIONS

Study on banana peel blackening during low temperature storage can be concluded as following:

1. Banana cv. Namwa was less sensitive to CI than cv. Hom Thong when stored at 4 and 12ºC.

2. Peel blackening of both cultivars was positively correlated with the LOX activity and level of lipid peroxidation products measured as TBA-reactive compounds.

3. Peel blackening of cvs. Hom Thong and Namwa banana correlated with the increased electrolyte leakage and PPO activity, combined with the decreased ratios of unsaturated / saturated fatty acids whereas total free phenolics and POD activity were not correlated with peel blackening.

4. Peel blackening of both cultivars during storage at 4ºC was delayed by hot water treatment at 42ºC for 15 min and had effect on increasing in the ratios of unsaturated / saturated fatty acids while there was no correlation in cv. Namwa with HW treatment.

5. HW treatment had no significant effect on total free phenolics and POD activity in cvs. Hom Thong and Namwa.

6. HW treatment had effect on reduced mRNA abundance of a gene encoding a *PPO* and also reduced the activities of both PPO and LOX, while increased the abundance of a *Hsp70* transcript in cv. Hom Thong and no changes in gene expression were detectable in cv. Namwa.

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APPENDIX