การเปลี่ยนแปลงในระบบแอนติออกซิแดนต์และการแสดงออกของยีนสร้างเอนไซม์แอนติออกซิแดนต์ในผลกล้วยหอมทองที่ผ่านการจุ่มน้ำร้อนในระหว่างการเก็บรักษา

นางสาว นิตยา อัมรัตน์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CHANGES IN ANTIOXIDANT SYSTEM AND EXPRESSION OF GENES ENCODING ANTIOXIDANT ENZYMES IN HOT WATER-TREATED 'HOM THONG' BANANA FRUIT DURING STORAGE

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Thesis Title	CHANGES IN ANTIOXIDANT SYSTEM AND EXPRESSION OF GENES ENCODING ANTIOXIDANT ENZYMES IN HOT WATER-TREATED 'HOM THONG' BANANA FRUIT DURING STORAGE
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นิตยา อัมรัตน์ : การเปลี่ยนแปลงในระบบแอนติออกซิแดนต์และการแสดงออกของยีนสร้างเอนไซม์แอนติออกซิแดนต์ในผลกล้วยหอมทองที่ผ่านการจุ่มน้ำร้อนในระหว่างการเก็บรักษา. (CHANGES IN ANTIOXIDANT SYSTEM AND EXPRESSION OF GENES ENCODING ANTIOXIDANT ENZYMES IN HOT WATER-TREATED 'HOM THONG' BANANA FRUIT DURING STORAGE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร. กนกวรรณ เสรีภาพ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.ดร. สายชล เกตุษา, Tracie K. Matsumoto, Ph.D., 130 หน้า.

ศึกษาผลของการจุ่มน้ำร้อนต่อการทำงานระบบแอนติออกซิแดนต์ในผลกล้วยหอมทอง (*Musa acuminata*, AAA group) โดยจุ่มผลกล้วยหอมทองในน้ำร้อนอุณหภูมิ 50 องศาเซลเซียส เป็นเวลา 10 นาที ก่อนการเก็บรักษาที่อุณหภูมิ 25 องศาเซลเซียส เป็นเวลา 10 วัน หรือ ที่อุณหภูมิ 14 องศาเซลเซียส เป็นเวลา 8 วัน ก่อนการเก็บรักษาที่ อุณหภูมิ 25 องศาเซลเซียส เป็นเวลา 8 วัน พบว่า กล้วยหอมทองที่เก็บรักษาที่อุณหภูมิ 25 องศาเซลเซียส มีการสุกในวันที่ 8 ถึงวันที่ 10 ของการเก็บรักษา โดยมีแนวโน้มของการชะลอการสุกในชุดที่มีการจุ่มน้ำร้อนก่อนการเก็บรักษา ซึ่ง พบการชะลอการเพิ่มขึ้นของค่าความสว่างของสีเปลือก (L value) การชะลอการลดลงของค่า hue และการชะลอการลดลงของความแน่นเนื้อในระหว่างการเข้าสู่ภาวะการสุก ระดับของ H_2O_2 ในกล้วยที่ผ่านการจุ่มน้ำร้อนมีแนวโน้มสูงกว่าชุดควบคุมในช่วงเริ่มเก็บรักษาแต่ลดต่ำกว่ากล้วยในชุดควบคุมในช่วงหลังของการเก็บรักษา ระดับของ MDA ในกล้วยที่ผ่านการจุ่มน้ำร้อนต่ำกว่ากล้วยในชุดการทดลองควบคุม การจุ่มน้ำร้อนไม่มีผลต่อการรั่วไหลของประจุไฟฟ้าของเซลล์ในระหว่างการเก็บรักษาแต่มีแนวโน้มของการลดต่ำลงในช่วงที่กล้วยสุก นอกจากนี้การจุ่มน้ำร้อนมีผลเล็กน้อยต่อระดับของวิตามินซี (AA) คาโรทีนอยด์ GSH และ อัตราส่วนของ GSH/GSSG ในช่วงที่กล้วยเข้าสู่ภาวะสุก ปริมาณของสารประกอบฟีนอลลิค ฟลาโวนอยด์ ในกล้วยที่ผ่านการจุ่มน้ำร้อนสูงกว่าชุดควบคุมในช่วงก่อนการสุก แต่ไม่มีความแตกต่างในผลกล้วยสุก การจุ่มน้ำร้อนมีแนวโน้มช่วยรักษาระดับการทำงานของสารแอนติออกซิแดนต์ แต่ไม่พบความแตกต่างทางสถิติ นอกจากนี้พบว่า มีการกระตุ้นของยีน *CAT* หลังการการจุ่มน้ำร้อน 1 และ 2 ชั่วโมง รวมถึงช่วงแรกของการเก็บรักษาและช่วงที่กล้วยสุก แต่ไม่พบการแสดงออกของยีน *SOD* จนกระทั่งวันที่ 4 และ 6 ของการเก็บรักษา ซึ่งแสดงให้เห็นว่า ยีน *CAT* มีบทบาทสำคัญในระหว่างการเก็บรักษากล้วยหอมทองที่อุณหภูมิ 25 องศาเซลเซียส มากกว่ายีน *SOD* ส่วนชุดการทดลองที่เก็บรักษากล้วยหอมทองที่อุณหภูมิ 14 องศาเซลเซียสก่อนการเก็บรักษาที่อุณหภูมิ 25 เซลเซียส พบว่า สามารถยืดอายุการเก็บรักษาผลกล้วยหอมทองได้ โดยการสุกเกิดขึ้นในระหว่างวันที่ 14 ถึง 16 ของการเก็บรักษา ซึ่งมีแนวโน้มของการชะลอการสุกในชุดที่ผ่านการจุ่มน้ำร้อน การจุ่มน้ำร้อนมีผลต่อการลดลงของ H_2O_2 แต่มีผลต่อ การเพิ่มสูงขึ้นของ MDA และการรั่วไหลของประจุไฟฟ้าของเซลล์ การจุ่มน้ำร้อนมีผลเล็กน้อยต่อการเปลี่ยนแปลงของวิตามินซี กลูตาไธโอนสารประกอบฟีนอลลิคและฟลาโวนอยด์ แต่ไม่มีผลต่อระดับการทำงานของ สารแอนติออกซิแดนต์ การแสดงออกของยีน *CAT* เพิ่มสูงกว่าชุดควบคุมหลังการเก็บรักษาที่ 25 องศาเซลเซียสเป็นเวลา 2 วันและในช่วงที่มีการสุก แต่ในยีน *SOD* พบว่าถูกกระตุ้นการแสดงออกด้วยการจุ่มน้ำร้อนหลังย้ายจากการเก็บรักษาที่อุณหภูมิ 14 องศาเซลเซียสจนกระทั่งวันที่ 2 ของการเก็บรักษาที่อุณหภูมิ 25 องศาเซลเซียส แต่การแสดงออกลดต่ำลงในช่วงที่เข้าสู่การสุก จากผลการทดลองแสดงให้เห็นว่า การจุ่มน้ำร้อนกระตุ้นการทำงานของระบบแอนติออกซิแดนท์ โดยเพิ่มระดับของสารแอนติออกซิแดนท์ เช่น กลูตาไธโอน สารประกอบฟีนอลลิคและฟลาโวนอยด์ นอกจากนี้ยังมีผลต่อการกระตุ้นของการแสดงออกของยีนสร้างเอนไซม์ *CAT* และ *SOD* โดย *CAT* จะถูกกระตุ้นการแสดงออกทันทีหลังการจุ่มน้ำร้อนและในช่วงที่กล้วยมีการสุก ส่วน *SOD* จะถูกกระตุ้นก่อนกล้วยเข้าสู่กระบวนการสุก

สาขาวิชา วิทยาศาสตร์ชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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NITTAYA UMMARAT: CHANGES IN ANTIOXIDANT SYSTEM AND EXPRESSION OF GENES ENCODING ANTIOXIDANT ENZYMES IN HOT WATER-TREATED 'HOM THONG' BANANA FRUIT DURING STORAGE. THESIS ADVISOR: ASST.PROF. KANOGWAN SERAYPHEAP, Ph.D., THESIS CO-ADVISOR: PROF. SAICHOL KETSA, Ph.D., TRACIE K. MATSUMOTO, Ph.D., 130 pp.

The effects of hot water treatment on antioxidant system were investigated in 'Hom Thong' banana (*Musa acuminata*, AAA group) fruits by immersing fruits in hot water (50 °C) for 10 min, before storage at 25 °C for 10 days or 14 °C for 8 days followed by storage at 25 °C for 8 days. Fruit ripening occurred on day 8 and up to day 10 of storage at 25 °C. According to L value, hue value and pulp firmness, hot water treatment showed a delaying of ripening during day 8 to day 10. The level of H_2O_2 were reduced in hot water treated banana during storage and ripening, although there was a tendency towards an increase of H_2O_2 in hot water treatment after treatment on day 0 and day 2. MDA contents in hot water treatment were also reduced throughout storage time. For electrolyte leakage, hot water treatment had minimal effect on the increase in electrolyte leakage after treatment and during early period of storage. However, the levels of electrolyte leakage in treated fruits decreased during ripening, compared with control. Regarding antioxidant compounds, hot water treatment showed slight effects on AA contents at early period of storage, while it tended to activate caroteniods and GSH contents, and the ratio of GSH/GSSG during fruits approaching ripening. According to phenolics, flavonoids and DPPH radical scavenging activity, the most active compounds were presented in free extracts. Hot water treatment affected phenolics at the beginning to storage but had no significant effect during fruit ripening. Furthermore, flavonoids increased in hot water treated fruit before ripening. The DPPH radical scavenging activity decreased during storage. Hot water treated fruits tended to have higher levels than those of control, although there was no significant difference between the treatments. Hot water treatment can activate *CAT* after treating (0 h and 1 h), during early periods of storage and also during fruit ripening. However, there was no effect on *SOD* expression after treatment. The activation of *SOD* can be observed on day 4 and day 6 of storage, and then it dropped during fruit ripening. Therefore, *CAT* seems to be an important gene that was induced by hot water treatment during storage at 25 °C. For banana fruit stored at low temperature (14 °C) before being transferred to 25 °C, the ripening was delayed in both control and hot water treatment. Ripening could be observed on day 14 up to day 16 of storage, which delayed ripening was found in hot water treated fruits. H_2O_2 contents in hot water treated fruits were slightly increased before fruit ripening, while the control fruit showed a sharp increase. However, MDA contents and electrolyte leakage in hot water treated fruit tended to be higher than those in control after being removed from 14 °C. Hot water treatment had minor effect on caroteniods, AA and glutathione contents. The increase of free phenolics and flavonoids in hot water treated fruits were higher than those of control during storage, although there was no significant difference during fruit ripening. Moreover, hot water treatment had no effect on antioxidant capacity throughout storage. *CAT* expression in hot water treatment was reduced after being removed from 14 °C, and then increased after 2 day of storage at 25 °C. When fruit began to ripen, hot water treated bananas exhibited higher *CAT* expression than in control fruits. *SOD* expression was induced when fruit was removed from 14 °C and showed continuously higher expression in hot water treated fruits up to day 12 of storage. However, lower *SOD* expression was found in hot water treatment when fruit ripened. These results suggest that hot water treatment can trigger antioxidant system as indicated by an increase of antioxidant compounds, including glutathione, phenolics and flavonoids, at early period of storage. Also, *CAT* tended to be activated immediately after treatment and during fruit ripening, whereas *SOD* showed a significant effect before fruit began to ripen.

Field of Study : Biological Science ..... Student's Signature .....

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LIST OF ABBREVIATIONS

AA	ascorbic acids
ABA	abscisic acid
APX	ascorbate peroxidase
CAT	catalase
CHS	chalcone synthase
CI	chilling injury
CM	chorismate mutase
DCIP	2, 6-dichlorophenolindolphenol
DHA	dehydroascorbic acid
DNPH	dinitrophenylhydradzine
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DTNB	5, 5-dithiobis-2-nitrobenzoic acid
EF-1α	elongation factor-1α
EtOH	ethanol
GR	glutathione reductase
GSH	glutathione (reduced form)
GSSG	glutathione (oxidized form)
H_2O_2	hydrogen peroxide
HSPs	heat shock proteins
HWT	hot water treatment
LOX	lipid peroxidase, lipoxygenase
MaExp	*Musa acuminata* expansin
1-MCP	1-methyl cycropropene
MDA	malonyldialdehyde

MeJA	methyl jasmonate
$NaNO_2$	sodium nitrite
PAL	phenylalanine ammonia-lyase
Pas	proanthocyanidins
PCR	polymerase chain reaction
PG	polygalacturonase
PL	pectate lyase
POD	peroxidase
PPO	polyphenol oxidase
SA	salicylic acid
SOD	superoxide dismutase
SSA	sulphosalicylic acid
ROSs	reactive oxygen species
TA	titratable acidity
TBA	thiobarbituric acid
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TDF	total dietary fiber
TSS	total soluble solid
$O_2^{\bullet-}$	superoxide radical

CHAPTER I

INTRODUCTION

'Hom Thong' banana (*Musa acuminata,* AAA group) is one of the most economically important fruit of Thailand. The limiting factors for exporting to distant countries are the short shelf-life and high sensitivity to chilling injury. Many postharvest treatments such as cold storage, some chemicals such as ethylene inhibitors, and heat treatment have been applied to prolong the shelf-life of the banana.

Postharvest heat treatments of fruit are used for insect disinfestation, disease control, and enhancement of fruit tolerances to other stresses and also maintenance of fruit quality during storage. Jintana Chancharoenrit (2002) found the effects of hot water dipping in delaying some physiological changes of 'Hom Thong' banana such as softening of fruit, increasing of total soluble solids (TSS), changing of peel color, and also decreasing activity of polyphenol oxidase (PPO), which all of that resulted in an increased storage life. Furthermore, we also discovered the effects of hot water treatment on activating some antioxidant enzyme activities including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) in banana peel (Nittaya Ummarat, 2005).

Several studies have identified genes expression during ripening of banana (Caamal-Vela´zquez, et al., 2007; Mbéguié-A-Mbéguié et al., 2007). However, the expression of gene encoding antioxidant enzymes in 'Hom Thong' banana, especially in hot water-treated banana is still not known. Therefore, in this research, we would investigate the effects of hot water treatment on antioxidant system including the levels of some non-enzymatic antioxidants and also the expression of genes associated with antioxidant enzymes that may be involved in banana fruit ripening during storage. Moreover, the effects of hot water treatment on the levels of oxidative stress were investigated.

The results of these investigations together with the relationship between hot water treatment on physiological changes of banana and antioxidant system responses will lead us to better insight about the mechanism of hot water treatment in banana fruit during storage and ripening.

Objectives:

1. To examine the effects of hot water treatment on the levels of oxidative stress in 'Hom Thong' banana fruit during storage.

2. To investigate the effects of hot water treatment on non-enzymatic antioxidant system in banana fruit during storage.

3. To determine the effects of hot water treatment on the expression of genes encoding antioxidant enzymes in banana fruit during storage.

Expecting benefits:

This research will lead to further understanding how hot water treatment can delay senescence of banana fruit during postharvest storage regarding to the non-enzymatic and enzymatic antioxidant system. Moreover, it will be useful for further molecular studies of banana and also for other fruits in order to preserve quality and delay fruit ripening according to antioxidative defense system.

Content of the thesis:

1. Literature review
2. Hot water treatment and measurement of fruit quality parameters
3. Determination of H_2O_2, MDA and electrolyte leakage
4. Determination of non-enzymatic antioxidants
5. Determination of the expression of *CAT* and *SOD*
6. Results and discussion
7. Conclusions

CHAPTER II

LITERATURE REVEIWS

1. Banana

Banana (*Musa* spp.) is a climacteric fruit which have high economical importance. It is considered a high energy food source with plenty of antioxidant vitamins and minerals. As a result, it has become one of the most important fruit crop in many developing countries, in terms of production and consumption (Manrique-Trujillo et al., 2007). Bananas are considered the fourth most important food crop in the world after three main cereals, rice, wheat, and corn. The world production of banana and plantain is about 88.5 million tons (Sukuntaros Tadakittisarn et al., 2009), including 'Hom Thong' banana (*Musa acuminate*, AAA group, cv Gros Michel) which is a commercially important cultivar in Thailand. However, a short shelf-life and high susceptibility to suffering severe postharvest losses of banana fruit are considered to be the limiting factors for a commercial production, especially in transglobal shipping. Therefore, better understanding of the banana ripening process and postharvest treatment are needed to improve fruit quality and prolong shelf-life.

2. Banana fruit ripening

Fruit ripening is genetically programmed, which is controlled by gene expressions. As climacteric fruit ripens, there occurs a sharp increase of respiration and ethylene production, loss of chlorophyll as a result of peel degreening, a production of volatile compounds making fruit become more aromatic, a conversion of starch to sugars resulting in more sweet and softer fruit (Manrique-Trujillo et al., 2007; Sukuntaros Tadakittisarn et al., 2009). In banana fruit, an increase in ethylene is needed to stimulate full ripening.

Moreover, the ripening process is associated with the increase in enzymes that are mostly involved in carbohydrate metabolism and cell wall degradation (Manrique-Trujillo et al., 2007). The increase in polygalacturonase (PG) and pectate lyase (PL) activity during banana ripening were directly correlated with the ripening of bananas. Activity of banana PG was maximized when fully ripened and reduced slightly when ripening progressed further. PL activity continuously increased during ripening progress (Sukuntaros Tadakittisarn et al., 2009).

According to CSIRO standards (1972), the ripening of banana can be categorized into 8 stages as followed (Sukuntaros Tadakittisarn et al., 2009):

Stage 1 = 100% green peel, hard fruit, no indications of ripeness

Stage 2 = 95% green peel, 5% yellow peel emerging

Stage 3 = 70% green peel, 30% yellow peel emerging

Stage 4 = 30% green peel, 70% yellow peel

Stage 5 = 95% yellow peel, 5% green at the tip

Stage 6 = 100% the whole fruit yellow (ripe)

Stage 7 = Yellow peel with brown spots appearing, characteristic aroma distinct (fully ripe)

Stage 8 = Yellow peel with more brown spots over ripe, pulp softens and pungent aroma (over ripe)

'Hom Thong' flesh at stage 6 to 8 is widely used for fresh consumption and for food ingredients such as banana juice and banana syrup. Somruedee Thaipanit and Pranee Anprung (2008) found that the stage 6 of 'Hom Thong' flesh had lower antioxidant activity than that of the stage 8. Thus, the higher ripening stage of the banana fruit has more antioxidant activity. Pokorny et al. (2001) had examined the carotenoid composition of banana flesh and found that there was a reduction in total carotenoid content in the flesh during the early stages of ripening, followed by carotenoid biosynthesis at the 6^{th} to 8^{th} of ripening stage. Carotenoid has antioxidant property. Analysis of the carotenoid composition in banana flesh during fully ripen revealed that the major types of carotenoids are α-carotene, β-carotene and lutein.

It was found that the higher flesh maturity stage of 'Hom Thong' exhibited significantly increased in the antioxidant activities, moisture content and reducing sugar, while the titratable acidity (TA) were significantly decreased. A significant decrease in TA, which was observed from stage 6 to stage 8, was due to the increase in respiration rate of the fruit at postharvest. In the respiratory process, many acids are substrates of Krebs cycle; thus, more respiration affects lower acid content (Seymour et al. 1993). It was reported that the main acid in bananas were citric, malic and oxalic (Hulme, 1970). However, total dietary fiber (TDF) contents showed no significant changes at different maturity stages (Somruedee Thaipanit and Pranee Anprung, 2008).

Several studies found that the moisture content or water content was significantly different at the different stages of ripening, which was gradually increased in moisture during ripening progresses. The rise in the moisture content of the 'Hom Thong' flesh during ripening is partly linked to the respiratory breakdown of carbohydrates (Somruedee Thaipanit and Pranee Anprung, 2008). John and Marchal (1995) reported that an osmotic migration of water from peel to pulp could

affect the increase of sugar concentration in the pulp (Somruedee Thaipanit and Pranee Anprung, 2008).

The lower pH in fruit indicates a more sour fruit. The increase of pH can also be observed during banana ripening, which can affect the taste of the fruit. Moreover, the volatile compound is a main factor affecting fruit aroma or flavor during ripening. In 'Hom Thong' banana, esters, alcohols, aldehydes and ketones are important volatiles. The major volatiles were 3-methylbutyl butanoate, 3-methyl-1-butyl acetate, 2-methyl-1-propyl butyrate, 2-methylpropyl ethanoate and 3-methylbutyl 3-methylbutanoate (Somruedee Thaipanit and Pranee Anprung, 2008).

The postharvest problems of banana fruit in relation to a high percentage of product loss are generally observed to stem from very limited shelf-life of fruit and an exogenous ethylene treatment that is not necessarily adapted. These postharvest problems include the "mixed-ripe" and/or "ship-ripe" fruit during transit, wound anthracnose, which is the main postharvest disease that develops during storage and fruit ripening, and also rapid ripening process (Mbéguié-A-Mbéguié et al., 2007).

3. Low temperature storage of banana fruit

Banana fruits are highly susceptible to chilling injury during low temperature storage. Chilling injury can be observed when store them at low temperature (less than 13 °C). It has been suggested that an early response of chilling injury is cellular membrane damage. Storage at low temperature may result loss of function of membrane-associated proteins; including ethylene receptors (McCollum and McDonald 1991; Fluhr and Mattoo 1996; Marangoni et al. 1996). The symptoms of chilling injury are, for instance, abnormal development of yellow skin color and failure of the fruit to soften (Satyan et al. 1992). Storage life of banana fruit is limited by the development of chilling injury symptoms when fruit are stored at temperatures of less than 13 °C. Darkening of the skin during cold storage is a common chilling injury symptom in banana fruit (Jiang et al., 2004). This phenomenon is considered as limiting factor in extending the storage life of the fruit, and is responsible for substantial postharvest losses in many areas of cultivation.

3.1 Roles of PPO and PAL during low temperature storage

The phenylpropanoid pathway is considered to be one of the most important metabolic pathways due to its responsibility for the synthesis of a large range of natural products in plants, including lignans, lignin, flavonoids and anthocyanins (Nugroho et al., 2002). The first step towards the phenylpropanoid biosynthetic

pathway is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), which converts L-phenylalanine to *trans*-cinnamic acid. L-phenylalanine is derived from chorismate by chorismate mutase (CM, EC 5.4.99.5) which involves prephenate as an intermediate (Figure 2.1).

PAL converts phenylalanine to mono- and diphenols, which are substrates of PPO (Tomas-Barberan et al., 1997). Increased PAL activity has been related to stress-induced disorders, including CI (Parkin et al., 1989). Sanchez-Ballesta et al. (2000) found that low temperature (2°C) can induce damage in the fruit flesh of citrus. This damage was related to the increase of PAL activity and also the accumulation of PAL mRNA. Hisaminato et al. (2001) suggested that PAL was involved in browning and the increase in PAL during chilling may also relate to a defense mechanism.

Oxidation of phenolic substrates by polyphenol oxidase (PPO) is believed to be a major cause of the brown discoloration of many fruits and vegetables (Walker and Ferrar, 1998). The degree of browning in banana, after cutting, was correlated with PPO activity and the concentration of free phenolic substrates (Jayaraman et al., 1982). According to Yang et al. (2001) the main PPO in banana peel is catechol oxidase (EC 1.10.3.1, o-diphenol: oxygen oxidoreductase). PPO enzymes have often been found to localize to the chloroplasts, cytoplasm and in vesicles between the plasmalemma and cell wall (Obukowicz and Kennedy, 1981). Free phenolics are present mainly in the vacuole, but are synthesized in the cytoplasm. It is believed that they may become deposited in the cell walls (Walker and Ferrar, 1998). When CI induces membrane damage of organelles such as vacuoles, the vacuolar phenolics may become decomposed by PPO. Alternatively, the cells may react to chilling by depositing phenolic compounds in the cell walls, by activating of PPO presented in the apoplast (Nguyen et al., 2003).

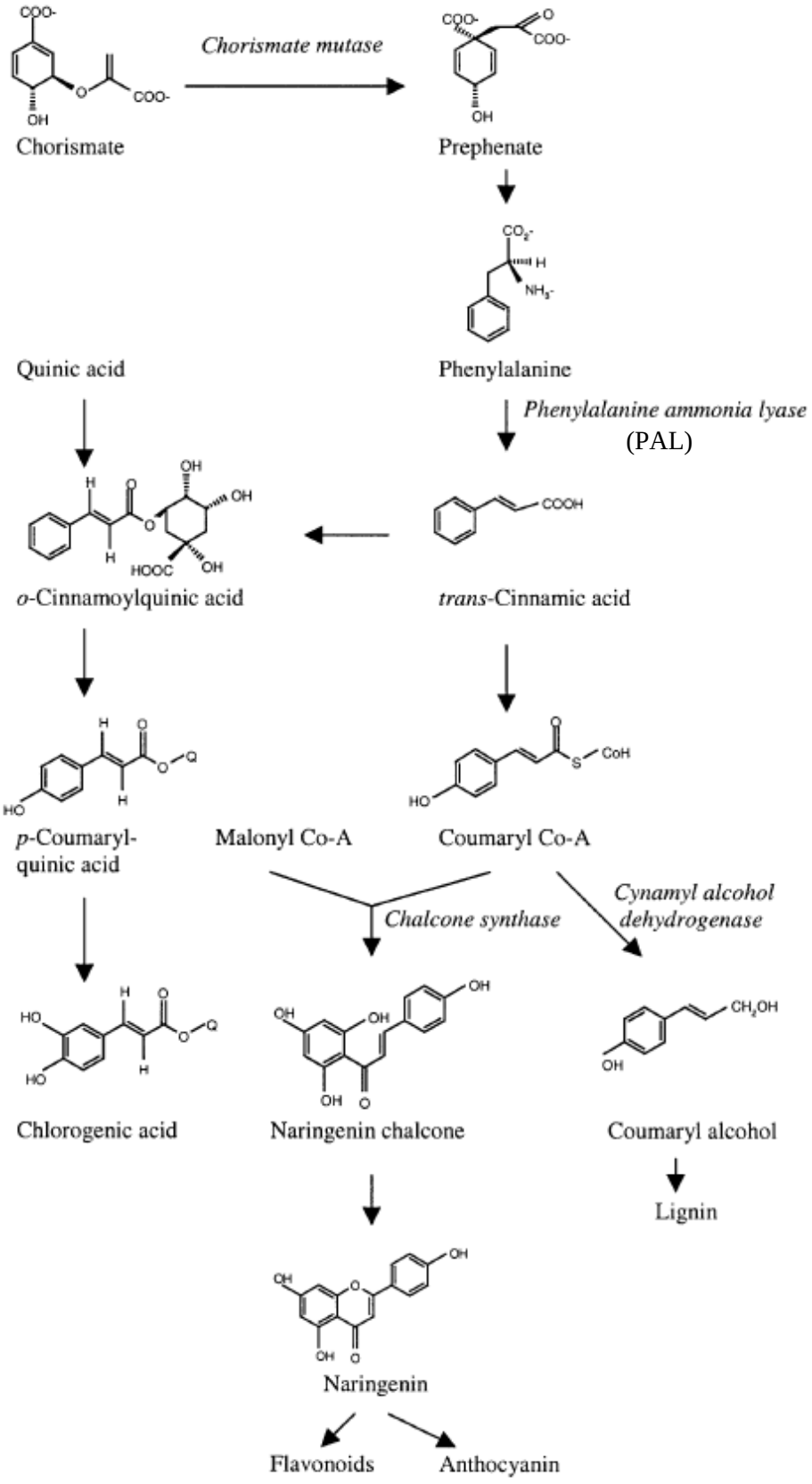


Figure 2.1 Pathways for the biosynthesis of phenylpropanoid products derived from phenylalanine (Nugroho et al., 2002).

Nguyen et al. (2003) reported the effects of low temperature storage on chilling injury of 'Kluai Khai' (*Musa* AA group) and 'Hom Thong' (*Musa* AAA group) bananas. They found that the visible chilling injury (CI) in the peel, mainly browning, occurred at 6 and 10 °C, but more sever at 6 °C, not different between cultivars. At the time of harvest, 'Kluai Khai' peel contained lower total free phenolics, lower PAL activity but higher PPO activity than those of 'Hom Thong' banana peel. When CI occurred, PAL and PPO activities in the peel increased, whereas total free phenolics decreased. The decrease in total free phenolic compounds and the increase in PAL and PPO activities occurred more rapidly at 6 °C than at 10 °C, in both banana cultivars. Correlations between visible CI and the level of total free phenolics, and between CI and the activities of PPO and PAL, were all highly significant. The results can suggest that low temperature stress can activate the activities of PAL and PPO, which resulted in browning. As in both cultivars, the concentration of total free phenolic compounds decreased during storage, this decrease was correlated with the degree of browning. Therefore, the free phenolic compounds may have been used as substrates for the browning reaction.

A strong inverse relationship was also observed between PAL activity and total free phenolics. The turnover of free phenolic compounds (by PPO and other enzymes) was apparently more rapid than free phenolic synthesis. Temperatures that induce CI in banana peel apparently activate PAL. This has also been described in other cases of CI, for example in mandarin peel (Martinez-Tellez and Lafuente, 1997). However, it is not clear whether the increase in PAL activity in banana peel occurs at the level of transcription or translation, or after translation (Nguyen et al., 2003).

Gooding et al. (2001) isolated a number of PPO (catechol oxidase) cDNA clones from banana fruit and found that levels of the PPO transcripts were low throughout ripening, in both flesh and peel. This result suggested that the browning of banana fruits during ripening resulted from the release of the pre-existing PPO enzyme, which is synthesized very early during fruit development.

Promyou et al. (2008b) found that storage at 4 °C can cause peel blackening on day 2 of storage in 'Hom Thong' banana and 2 days later in 'Namwa' banana. The increase of lipoxygenase (LOX) and catechol oxidase (polyphenol oxidase, PPO) activities were also observed during low temperature storage, which is correlated with peel blackening in 'Hom Thong' banana, but not 'Namwa'. However, levels of total free phenolics, thiobarbaturic acid (TBA)-reactive compounds, and peroxidase (POD) activity in the peel were not correlated with blackening in both cultivars.

3.2 Roles of ethylene during low temperature storage

Storage at low temperatures (<11 °C) results in CI as shown by peel pitting and discoloration and abnormal fruit ripening. CI of banana fruit appears to be associated with suppressed ethylene binding capacity resulting in a failure of fruit ripening and softening (Jiang et al., 2004).

Ripening of climacteric banana fruit is associated with a sharp increase in ethylene evolution. Ripening is initiated either by the natural evolution of endogenous ethylene as banana fruit reach full maturity or by using commercial exogenous ethylene ripening procedures (Will et al, 2001). It is considered that ethylene binds to its receptors and elicits a signal transduction process that leads to normal fruit ripening (Fluhr and Mattoo, 1996).

Jiang et al. (2004) reported that the skins of banana fruit stored at 3 and 8 °C showed chilling injury symptom, peel darkening, as storage duration increased. This chilling effect was correlated with the loss of membrane permeability as shown by increased relative electrolyte leakage from skin tissue. Whereas, banana fruit stored for 8 days at 13 °C (optimum temperature) showed no chilling injury symptoms. Although, exposure of banana fruit to the ethylene binding inhibitor 1-methylcyclopropene (1-MCP), as a blocker of ethylene receptors prior to cold storage, can prevent ripening, this treatment also enhanced the chilling injury by accelerating the increase of membrane permeability. According to ^{14}C-ethylene release assay, it was found that application of 1-MCP and storage at low temperature affected on the decrease of ethylene binding ability, which is related to more sever chilling injury. Moreover, fruit exposed to 1-MCP for 12 h and then stored at 3 or 8 °C exhibited lower ethylene binding ability than those stored at 13 °C. Thus, chilling injury of banana fruit stored at low temperature is associated with a decrease in ethylene binding ability. This result supports the involvement of reduced ethylene binding in enhancing chilling injury symptoms in 1-MCP treated fruit. Therefore, it can suggest that chilling injury of banana fruit stored at low temperature is due to reduced ability of tissue to respond to ethylene.

3.3 Roles of expansin during low temperature storage

Fruit ripening is relatively complex. Disassembly of the primary cell wall is a primary process in fruit ripening and softening (Brummell and Harpster, 2001).

Expansins are cellular proteins considered to facilitate cell wall extension during plant growth, seed germination and fruit ripening (McQueen-Mason et al., 1992). Expansins have been identified from tomato, banana, peach, strawberry and

pear fruits and are associated with fruit maturation and softening (Hayama et al., 2003; Lee and Kende, 2001; Trivedi and Nath , 2004).

Wang et al. (2006) found that banana fruit treated with propylene (functional ethylene analogue) before storage at 7 °C showed CI symptoms after 7 days, while untreated control fruit observed after 4 days. The result suggested that application of propylene, which can stimulate ripening, tended to alleviate CI during storage at low temperature. The propylene treatment accelerated color change, increased ethylene production rate and caused a more rapid decrease in peel and pulp firmness after removal to 22 °C. According to RNA blotting analysis, it showed no accumulation of either *MaExp1* or *MaExp2* transcripts in banana fruit during low temperature storage. After transferred from cold temperature, expansin genes were expressed more intensively in propylene pre-treated fruit than in control. The results suggest that pre-treating with propylene can increase tolerance of fruit to chilling injury, which is related to higher ethylene production and increase expression of *MaExp1* and *MaExp2* after removal from cold temperature. These results suggested that expansin gene expression is involved in ripening and softening of banana fruit after storage at cold temperature. This observation is also in agreement with that of Zhou et al. (2001), who found that ethylene plays a positive role in alleviating CI of nectarines. However, ethylene production and fruit softening processes at low temperature were not coupled with expression of expansions.

4. Antioxidant system and fruit ripening

4.1 Enzymatic antioxidant system

Reactive oxygen species (ROSs) are the primary mediators of oxidative damage in plants, which is involved in fruit senescence (Halliwell and Gutteridge, 1989; Hariyadi and Parkin, 1991). Excessive generation of ROSs, such as superoxide anion and hydrogen peroxide, can be a significant factor in damage of tissue. There are numerous sites of ROSs production within cells including various cytochrome enzymes, xanthine oxidase, and the mitochondrial electron transport chain. These ROSs can rapidly attack all types of bio-molecules to cause membrane injury, lipid peroxidation, and DNA mutation, leading to structural dysfunctions, abnormal metabolic and finally cell death (Halliwell and Gutteridge 1989). Reactions involving activated oxygen species can promote a general deterioration of cell metabolism, resulting in senescence and fruit ripening.

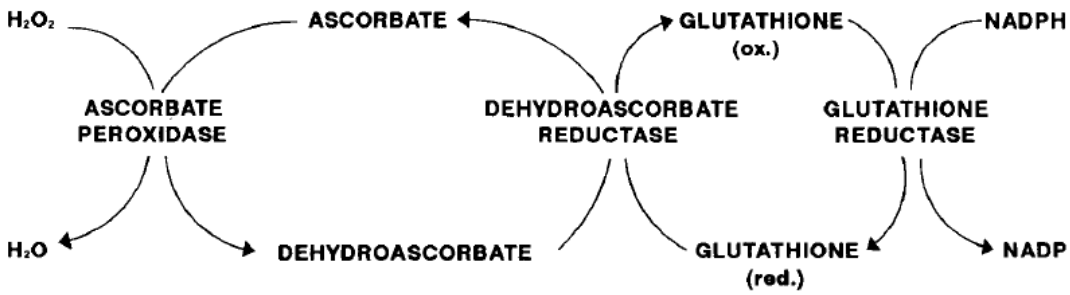


Figure 2.2 Halliwell-Asada Enzyme pathway (Price et al., 1994)

This pathway is involved in enzymes of scavenger metabolism, such as ascorbate peroxidase, glutathione reductase, and dehydroascorbate reductase, that together form the Halliwell-Asada pathway (Ox. = oxidation; red. = reduction).

Since the levels of ROSs increase dramatically during fruit ripening, changes in antioxidant system play an important role in the ripening process. To control the level of ROSs, plants have evolved several anti-oxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT), and non-enzymatic antioxidants such as ascorbate (vitamin C), α-tocopheral, carotenoids and glutathione (Jimenez et al., 2002; Juan et al., 2010). Jimenez et al. (2002) found that the levels of antioxidant enzyme activities, including SOD, CAT and the enzymes involved in the ascorbate-glutathione cycle were increased during ripening process and reduced as ripening progressed, these increases were associated with changes in mRNA levels of these enzymes.

The damage of plant tissues has been associated with reactive oxygen species (ROSs) arising under stress conditions such as exposure to ozone, chilling (Wise and Naylor, 1987) and water stress (Sala and Lafuente, 2004). Although an increase of ROSs, such as hydrogen peroxide (H_2O_2), can function as a signaling molecule that mediates responses to abiotic and biotic stresses (Neill et al., 2002), such as enzyme activation and gene expression, high production of ROSs, including superoxide and hydroxyl radicals, singlet oxygen and also hydrogen peroxide, may cause damage to the membrane lipids, proteins and nucleic acids thus disrupting the homeostasis of the organism (Kang et al., 2002) and lead to the death of the cells (Janda et al., 2003). To control the level of ROSs, these enzymatic antioxidants, and non-enzymatic antioxidants such as ascorbate (vitamin C), α-tocopheral, carotenoids and glutathione are adjusted to the changing ROSs concentration (Janda et al, 2003). Moreover, several studies have reported that plants could modify their phenolic metabolism by inducing the accumulation of phenolic compounds as a mechanism to protect cells against the oxidative damage (Lemoine et al., 2010). The balance between the active oxygen detoxifying enzymes, including SOD, APX, GR and CAT in cells is crucial

for determining the steady-state level of superoxide radicals and hydrogen peroxide. It has been reported that APX might be responsible for the fine modulation of ROSs for signaling, where as CAT might be responsible for the removal of excess ROS during stress (Sala and Lafuente, 2004). The superoxide radical ($O^{\bullet-}_2$) is dismutated to H_2O_2 by SOD (EC 1.15.1.1); and CAT (EC 1.11.1.6) and APX (EC 1.11.1.11) metabolize H_2O_2 to H_2O. APX requires ascorbate to function. GR (EC 1.6.4.2) and glutathione function in the regeneration of reduced ascorbate after it is converted to monodehydroascorbate by APX (Kang et al., 2002).

Chilling temperatures may induce oxidative stress in fruit tissues. The main difference between chilling-sensitive and chilling-tolerant cultivars are in the higher activity of the chilling tolerant to break down H_2O_2 by CAT activity and co-operation of APX and GR activities (Sala, 1998). In addition, it has been suggested that the CAT may be a major antioxidant enzyme involved in the defense mechanism of mandarin fruits against chilling stress (Sala and Lafuente, 2000). APX was highly induced by low temperature stress during storage of potato tubers and this enzyme also responds to wide ranges of environmental stress (Kawakami et al., 2002).

During fruit storage, the presence of wounds and fungal development constitutes another stress source that increases the amount of ROSs (Vicente et al., 2006). Many researchers have been focusing on the effects of these stresses on antioxidant genes. For instance, it was found that water deficit could activate the expression of *APX* gene (Jin et al., 2006). Salt stress can induce altered expression of *APX, CAT, GR* and *SOD* in rice seedling (*Oryza sativa* L.) that may result from disruption of cell redox homeostasis (Menezes-Benavente et al., 2004). Also the biotic stress induced by pathogen can cause the oxidative burst and induce the expression of *SOD* during infiltration with bacterial suspensions (Voloudakis et al., 2006).

4.2 Non-enzymatic antioxidant system

Beside enzymatic antioxidants, plant can also activate non-enzymatic antioxidants involved in the ascorbate-glutathione cycle such as ascorbate (vitamin C) and glutathione, α-tocopheral, carotenoids, flavonoid and phenolic compounds to decrease the damage resulting from ROSs (Babbar et al., 2011; Jimenez et al., 2002; Vijayakumar et al., 2008).

4.2.1 Ascorbate or ascorbic acids (AA)

Ascorbic acid (vitamin C) is a six carbonketo-lactone, a strong reducing agent, serves as an antioxidant and as a cofactor in hydroxylation reactions.

The hydrogen donation from ascorbic acid is thought to be primarily responsible for the anti-oxidant properties. It consists of four hydroxyls (two enol hydroxyls on carbon 2 and carbon 3) and a lactone, it is very easy to get oxidized and changes to dehydroascorbic acid (DHA), hydroxyls play a very important role as an antioxidant (Liu et al., 2006).

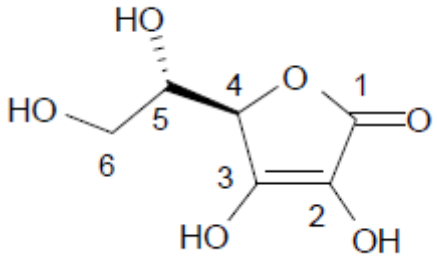


Figure 2.3 The structure of ascorbic acid (Liu et al. ,2006)

Ascorbic acid is a sugar acid with antioxidant properties. Its appearance is white to light-yellow crystals or powder, and it is water-soluble. One form of ascorbic acid is commonly known as vitamin C or L-ascorbic acid or L-ascorbate. L-Ascorbate has an important role in plant cells as an antioxidant molecule that prevents oxidative stress caused by photosynthesis, oxidative metabolism or exposure to pollutants (Smirnoff, 1996; Arrigoni et al., 1997; Loewus, 1999). Moreover, L-ascorbate and its oxidation product L-dehydroascorbate can act as an essential nutrient for humans, mostly presented in fruits and vegetables (Nascimento et al., 2005).

Ascorbate can be found in chloroplasts, cytosol and vacuole, and apoplastic space of leaf cells in high concentrations. It is considered to be the most important antioxidant in plants, with a fundamental role in the removal of hydrogen peroxide. Oxidation of ascorbate occurs in two sequential steps, first producing mono-dehydroascorbate, and if not rapidly re-reduced to ascorbate, the mono-dehydro-ascorbate disproportionates to ascorbate and dehydro-ascorbate (Arora et al., 2002). Besides being a potent antioxidant, ascorbate is involoved in the pH mediated modulation of PSII activity and its down-regulation associated with zeaxanthin formation. This potent mechanism is useful for preventing photo-oxidation (Arora et al., 2002).

4.2.2 Carotenoids

Carotenoids are isoprenoid molecules that are common to all photosynthetic tissues. They are divided into the hydrocarbon carotenes, such as lycopene and β-carotene or xanthophylls, typified by lutein (Bramley, 2002). They are one of antioxidants that can participate in light harvesting in photosynthetic membranes, and also protect the photosynthetic apparatus from excessive light energy by quenching triplet chlorophylls, superoxide anion radicals and singlet oxygen (Niyogi, 1999; Arora et al., 2002). Furthermore, they are considered as the essential components of some pigment-protein complexes (Moskalenko and Karapetyan, 1996). Parry et al. (1990) reported that carotenoids are precursors of abscisic acid.

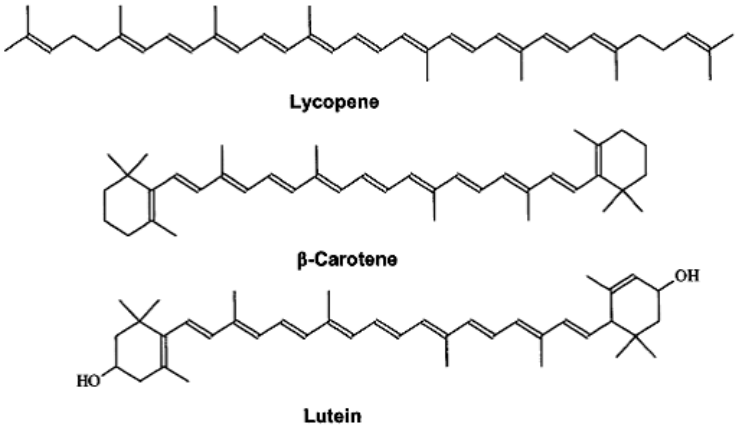


Figure 2.4 Structures of typical carotenoids (Bremley, 2002))

4.2.3 Glutathione

Glutathione, glutamyl cysteinyl glycine (GSH) is the major low molecular weight thiol compound in most plants. Glutathione acts as disulphide reductant to protect thiol groups on enzymes, regenerate ascorbate and react with singlet oxygen and hydroxyl radicals (Arora et al., 2002). It also participates in the regeneration of ascorbate from dehydro-ascorbate via the enzyme dehydro-ascorbate reductase. GSH can be oxidized to glutathione disulphide (GSSG). Then, GSH is regenerated by glutathione reductase (GR) in a NADPH-dependent reaction. Creissen *et al*. (1996) have reported that GR is located in chloroplastic, mitochondrial and cytosolic fractions of maize. Recent studies have further confirmed its existence in chloroplast, mitochondrial and cytosolic fractions (Gomez et al., 1999; Hernqdez et al., 2002).

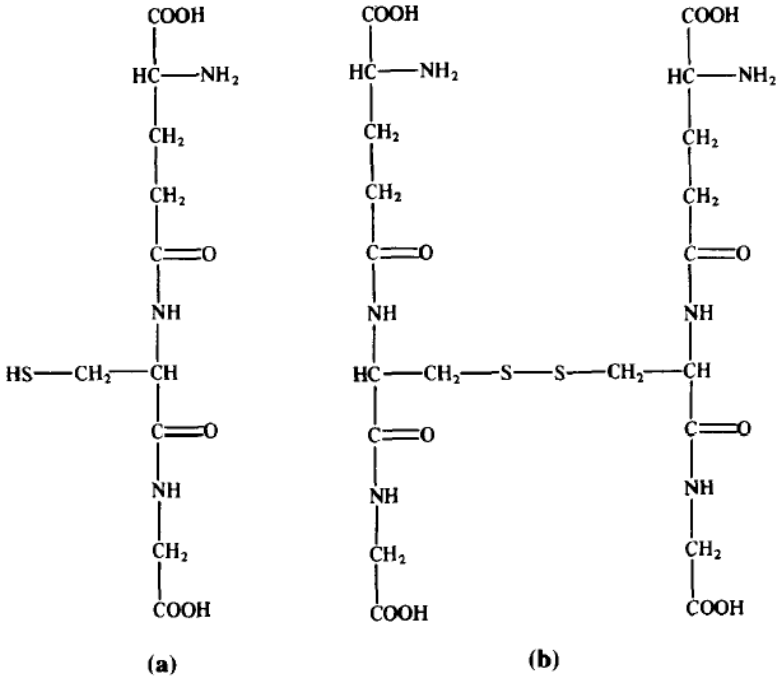


Figure 2.5 Structure formulae of reduced glutathione (GSH) (a) and oxidized glutathione (GSSG) (b) (Gennaro and Abrigo, 1992).

4.2.4 Phenolics

Phytochemicals, especially phenolics in fruit and vegetables are the major bioactive compounds known for the health benefits. Plant phenolics are commonly found in both edible and non-edible parts of the plants and have been reported to have multiple biological effects, including antioxidant activity. Phenolic compounds are believed to account for a major portion of the antioxidant activity in many plants (Babbar et al., 2011).

Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid, and a type of organic acid, found in plants. The chemical formula is $C_6H_2(OH)_3COOH$. Gallic acid is found both free and as part of tannins.

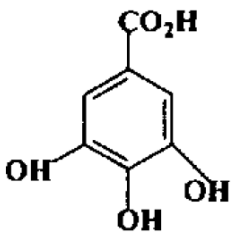


Figure 2.6 Structure of gallic acid (Salah et al., 1995)

The scavenging activity of phenolic is mainly due to their redox properties, which allows them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Babbar et al., 2011). Many natural antioxidants exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory action (Cook and Sammon, 1996). The antioxidant activity is not fully contributed by phenol alone. The other constituents such as ascorbates, reducing carbohydrate, tocopherols, carotenoids, terpenes and pigments as well as the synergistic effect among them could possibly contribute to the total antioxidant activity (Babbar et al., 2011). The preservation of fruit phenolic content had a great impact on the quality of fruits because of the contribution of phenols not only in enzymatic browning reactions but also on nutritional value of the product, as antioxidant capacity (Kervers et al., 2007). The phenolic compounds associated with lignin synthesis in damage pericarp of mangosteen fruit after impact, lignin content increased while total phonics decreased (Apita Bunsiri et al., 2003).

There are some reports about a transient increase in phenolic compounds during a few days of storage in plum, tomato, broccoli, black grape, while a transient decrease was observed in citrus, lettuce and celery. In banana, the phenolic contents decreased rapidly during storage (only 20% was still present after 2 days) (Kervers et al., 2007). In tomatoes, levels of aqueous phenolics increased during ripening. This increase correlated with an increase in total antioxidant activity, which mainly due to changes of the lipophillic antioxidant activity. On the other hand, the hydrophilic antioxidant remained practically unchanged during ripening (Cano et al., 2003).

During medlar fruit ripening, (ripe to over-ripe maturities), total phenolic contents were gradually decreased, while PPO activity increased (Ayaz et al., 2008). Moreover, the differences in harvest time, season, fruit maturity and genotype during ripening resulted in considerable effects of the distribution of PPO isoforms, activity, characterization and phenolics in medlar fruit (Ayaz et al., 2008). In addition, Gruz et al. (2011) reported that the concentration of phenolic acids mostly decreased as the ripening progressed. However, the insoluble ester-bound phenolics increased at the early stages of maturity and decreased only during the ripe to over-ripe stage of maturity.

In blueberry, antioxidant activity and total phenolic content tended to decrease during ripening. Additionally, different cultivation and climate condition can affect total phenolics (Castrejon et al., 2008). In acerola fruits (*Malpighia emarginata* D.C.), the phenolic compound content decreased during maturation (Lima et al., 2005). The decreases of phenolic level during the maturation process also were found in pear (Amiot et al., 1995) and apple fruits (Burda et al., 1990). In guava, ascorbic acid and phenolic compounds decreased continuously during ripening. The peel showed higher values of ascorbic acid, total proteins, and phenolic compounds

than the pulp (Bashir et al., 2003). Kondo et al. (2005) found that total phenolics of guava, mango and rose apple skin were higher than what found in the flesh. They were also high at the immature stage and then decreased towards ripening.

In banana, total phenolics were more abundant in peel than in pulp. This result was consistent with the antioxidant activity. The difference in the antioxidant activities between the peel extract and the pulp extract may be attributed to their phenolic contents (Someya et al., 2002). Moreover, gallocatechin was more abundant in peel than in pulp. The higher gallocatechin content of the banana (*Musa Cavendish*) peel may account for the better antioxidant effects (Someya et al., 2002). In contrast, Kondo et al. (2005) found that total phenolic of banana flesh were higher than in the skin.

4.2.5 Flavonoids

Flavonoids are polyphenolics with diphenyl propane (C6-C3-C6) skeleton. They are considered to be the largest group of naturally occurring phenols and it is estimated that 2% of all the carbon photosynthesized by plants is considered into flavonoids (Alothman et al., 2009).

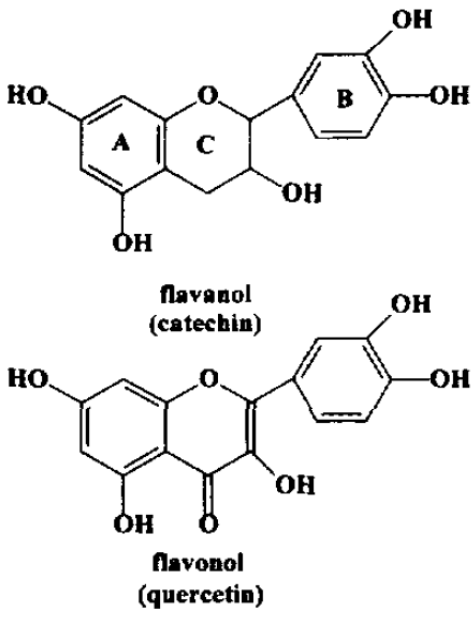


Figure 2.7 Structures of representative flavonol, catechin, and flavonol, quercetin (Salah et al., 1995)

Flavonoids are widely distributed plant secondary metabolites resulting from the addition of malonyl CoA to the phenylpropanoid molecule coumaroyl CoA (Figure 2.1) (Pourcel et al., 2006). These polyphenolic compounds are characterized by two aromatic cycles (A- and B- rings) linked by a heterocycle (C-ring) (Figure 2.7). They are classified according to the oxidation degree of the C-ring, and include flavonols, anthocyanins and flavan-3-ols. The condensation of flavan- 3-ols leads to the formation of proanthocyanidins (PAs), also called condensed tannins (Marles et al., 2003).

Flavonoids exhibit functions to response to stress environmental conditions. For example, they protect the plant against ultraviolet radiations. They also have antimicrobial properties and act as a deterrent for herbivores by limiting assimilation of dietary proteins and inhibiting digestive enzymes (Scalbert, 1991). Flavonoids also play important roles in human health through consumption of plant-derived foods, by preventing degenerative diseases associated with oxidative stress (Williams et al., 2004). Furthermore, flavonoids act as scavengers of free radicals such as reactive oxygen species (ROS), and also prevent their formation by chelating metals (Vaknin et al. 2005).

Several studies have emphasized that flavonoids from different botanical sources can act as powerful antioxidants, even more so than can the traditional vitamins (Vijayakumar et al., 2008). Anthocyanins have less antioxidant potential than other phenolic compounds such as flavonols which among the flavonoids are considered good antioxidants. However, it was found that antioxidant activity of fruits seems to be more influenced by genetic differences than physiological ripening changes (Castrejon et al., 2008). Maturation and ripening processes as well as climate conditions appeared to have a more pronounced effect on flavonoid biosynthesis and phenolic composition in comparison to genetic different of high bush blueberry varieties (Castrejon et al., 2008).

Quercetin is a typical flavonoid ubiquitously presented in vegetables and fruits (Kervers et al., 2007). Quercetin and anthocyanins have been showed to be strong antioxidants (Wach et al., 2007; Kahkonen et al., 2003). Flavoniods biosynthesis is tightly associated with the development stage of the fruit (Castrejon et al., 2008). In bilberry (*Vaccinium myrtillus*), a gene coordination expression of flavonoid biosynthesis is related to the accumulation of anthocyanins, proanthocyanidins and flavonols in developing fruit (Jaakola et al., 2002). In strawberry (*Fragaria* x *ananassa*), flavonoid biosynthesis has two distinct key flavonoid enzymes activity peaks during fruit ripening at early and late development stages (Halbwirth et al., 2006). In blueberry, an increase of anthocyanin content and a decrease of total phenolics during ripening can suggest a shift in the pool of total phenolics toward anthocyanin synthesis and an overall decline in the content of other phenolic components (Kalt et al., 2003).

Generally, the flavonoid content of fruits and vegetables increased during storage, or was stable. A transient increase in flavonol glycon and anthocyanin levels was observed in plum (Kervers et al., 2007), while the flavonoid and aglycones in banana decreased rapidly. Dupont et al. (2000) also found the loss of total flavonoid in banana and lettuce during storage. In blueberry, anthocyanins increased during harvest stage mean while flavonols and hydroxycinnamic acids decreased from unripe green to ripe blue stage of berry ripening (Castrejon et al., 2008).

In apple fruit, the most abundant flavonoids were found in the skin (Awad et al., 2001). Quercetin glycosides, phloridzin and chlorogenic acid reached the highest amount during early season, but decreased at different rates during fruit development to reach a steady level during maturation and ripening (Awad et al., 2001). These decreases might be due to a dilution of these compounds caused by the increase in the total amount per fruit during progressive growth.

4.2.6 Total antioxidant activity

Most of the bioactive antioxidant compounds were phenolics. A high correlation between total phenolic content and antioxidant capacity measured by the DPPH method had been reported (Kervers et al., 2007; Gruz et al., 2011). This significant correlation suggests that the presence of the phenolic compounds largely accounted for the antioxidant capacity measured with DPPH (Kervers et al., 2007). Moreover, the correlation between phenolic compound and antioxidant capacity obtained by ORAC measurements could be found in potatoes (Andre et al., 2007). However, there were some divergences exist between the results obtained with the ORAC assay and the DPPH method. For example, in banana, green grapes and lemons, which have the highest total phenolic contents, had high antioxidant capacity as assayed by only DPPH method, whereas, plums, oranges and cherries had higher antioxidant capacity when assayed by the ORAC assay (Kervers et al., 2007). Therefore, the radical source used in the assay can have dramatic effects on the result because of the differential response of different types of antioxidant compounds to the radical source (Cao et al., 1996).

Most fruits with high antioxidant capacity had also a high level of ascorbic acid (strawberries, bananas, lemons and oranges) (Kervers et al., 2007). In banana, it was found that banana skin had a higher DPPH radical scavenging activity compared to the flesh and contained more polyphenolics and ascorbic acid contents than the flesh. DPPH radical scavenging activity was associated with total phenolic concentration (Kondo et al., 2005). The high antioxidant capacity in banana (*Musa acuminate colla* AAA) peel extracts could be an indicator for a good lipid peroxidation inhibitor (Montelongo et al., 2010).

During storage, there are some reports suggested that in most fruits and vegetables the storage did not negatively affect the antioxidant capacity. However, a transient increase of the antioxidant capacity was measured as in yellow pepper, asparagus and plum (Kervers et al., 2007). In orange and apple, the antioxidant doubled rapidly and afterward was stable. In onion, the antioxidant capacity continuously increased during storage (> 10 times after 23 days) (Kervers et al., 2007). A decrease of antioxidant capacity during storage had been reported in apricot (25%), and decreased by more than 50% in spinach, banana, broccoli and leek (Bartolini et al., 2006; Kervers et al., 2007; Kondo et al., 2005). The decrease in DPPH radical scavenging activity during fruit maturation can suggest a decrease in natural antioxidants in fruits (Gruz et al., 2011).

Kondo et al. (2005) studied antioxidant activities and antioxidants of the tropical fruit guava (*Psidium guajava* L.), mango (*Mangifera indica* L.), banana (*Musa* spp.), rose apple (*Syzygium jambos* Alston), and papaya (*Carica papaya* L.). It was found that, in generally, IC_{50} values of superoxide (O_2^{-}) and DPPH radical scavenging activity in fruit are linked with total phenolics, but the DPPH IC_{50} in papaya was associated with AA concentrations.

According to the effect of low temperature on antioxidant activity and jasmonates in the skin of bananas and mangoes, degree of chilling injury was higher at 6 °C compared to 12 °C. Endogenous jasmonates, superoxide dismutase (SOD) activity, total phenolics, and AA were each associated to the degree of chilling injury. In general, IC_{50} values of O_2^{-} and DPPH radical scavenging activity were also correlated with the degree of chilling injury, except O_2^{-} IC_{50} in mango that showed no significant difference. These results can suggest the relations of jasmonates, AA, and polyphenolics to the chilling injury of tropical fruit during storage at low temperature (Kondo et al., 2005).

Kondo et al. (2004) reported that polyphenolic flavanones such as naringin and hesperidin failed to scavenge either O_2^{-} or DPPH radicals, although catechin and AsA scavenged both radicals. Carotenoids such as carotene, lycopene, and xanthophyll have been reported to quench singlet oxygen (Conn et al., 1991). Cryptoxanthin failed to scavenge DPPH radicals, but it had an effect on O_2^{-} (Kondo et al., 2004).

Fruit antioxidant activity decreases with the progress of senescence (Srilaong and Tatsumi, 2003). Although low temperature may extend storage life of tropical fruit, it also causes chilling injury, characterized by browning of the skin, greater firmness, and off-flavours in the fruit. The temperature that induces chilling injury differs with fruit species, and the skin is generally more sensitive to low temperature than the fruit flesh (Kondo et al., 2005).

Kondo et al. (2004) suggested that O_2^- IC_{50} may have been influenced by phenolic concentrations since O_2^- scavenging activity in (+)-catechin and (−)-epicatechin is four times as strong as that in AsA. DPPH radical scavenging activity of (−)-epicatechin is lower than that of (+)-catechin. However, phloridzin did not scavenge O_2^-or DPPH radicals.

Turner et al. (2002) found that jasmonates were associated with defense response in plant. Richard et al. (2000) reported that jasmonate treatment induced chalcone synthase (CHS) gene expression in white spruce (*Picea glauca*). CHS is an upstream enzyme in the phenylpropanoid pathway, which leads to the production of antioxidant substances such as lignin and flavonoids (Lancaster, 1992).

Increase of antioxidant activity and total phenolic contents were found in blueberries during cold storage (Connor et al., 2002) and a positive correlation between methyl jasmonate (MeJA) application and the increase of AA and of phenylalanine-ammonia lyase (PAL) activity, which is associated with a plant's defense system, has been observed in guava fruit (Gonzalez-Aguilar et al., 2004). These facts suggest a connection among jasmonates, AA, and polyphenolics in the inhibition of chilling injury.

Gonzalez-Aguilar et al. (2001) reported the effect of MeJA treatment on increase of sugar concentrations in mangoes and guava fruit stored at 5 °C that were still ripening. The increase of sugar in MeJA-treated fruit was considered to be a part of the plant defense response system. Moreover, MeJA treatment could decrease membrane-lipid peroxidation and maintained high SOD activity in strawberries (*Fragaria vesca*) during water stress (Wang, 1999). These results suggest a positive correlation among jasmonates, SOD activity, and O_2^- scavenging activity.

5. Heat treatment

Postharvest treatments have been used to preserve quality of fresh produce and have been focused mainly on preserving freshness. However, as a secondary response under certain adverse environmental and stress conditions has been observed, including some types of stress used as postharvest treatments.

Several postharvest treatments have been developed to preserve the quality of fresh produce, including ultraviolet light, controlled and modified atmospheres, edible coatings, heat treatments, and natural compounds, among others. Most postharvest treatments involve the alteration of the natural conditions of the fruit in order to prolong its postharvest life and avoid microbial growth. Moreover, some responses to postharvest treatment are involved in an improvement on the antioxidant system, which is a benefitial effect for products (Gonzalez-Aguilara et al., 2010).

However, postharvest treatments have many effects to products. For example, high O_2 atmospheres and irradiation cause damage to some vital molecules of food deteriorative microorganisms, in addition of altering some biochemical processes in the fruit (Charles et al., 2009). Heat treatments affect a wide range of fruit ripening processes such as ethylene synthesis, respiration, softening and cell-wall metabolism (Zhang et al., 2009).

Fruits and vegetables are living tissues subjected to quality changes after harvesting. There are three important aspects that conform the quality of fresh produce: (1) sensorial quality: aroma, firmness and colour ; (2) safety: pathogens and deteriorative microorganisms; and (3) nutritional value: content and bioavailability of bioactive compounds. Most of research has been done focusing on the effect of postharvest treatments on sensorial quality and safety of fresh-tropical fruits. However, reports on the changes of bioactive compounds after postharvest treatments of tropical fruits are scarce (Gonzalez-Aguilara et al., 2010).

Postharvest heat treatments have been used in fruit for insect disinfestations, disease control, ripening decay, ripening processes and modification of fruit responses to other stress (Paull and Chen, 2000; Vicente et al., 2006; Gonzalez-Aguilar et al., 2010). These effects depend on the type of heat treatment applied and duration of fruit exposure (Gonzalez-Aguilar et al., 2010) and also including species and even varieties (Lurie, 1998). It is possible to apply a moderate treatment at non-lethal temperatures resulting in both a reversible suspension of ripening and a reduction of fungal decay without noticeable changes in fruit quality (Lurie, 1998). Heat treatment affects several aspects of fruit ripening, such as ethylene production and cell wall degradation, probably through changes in gene expression and protein synthesis (Lurie, 1998). Some postharvest treatment can activate an antioxidant system as a response to stress, resulting in improving the antioxidant activity of fruits (Gonzalez-Aguilar et al., 2010). These results indicate the efficiency of heat treatment in increase postharvest life of some fruits and induce an increase of bioactive compounds.

Extensive commercial application of heat treatments has emerged over the past two decades and has been used for disinfestations and disinfection of various tropical fruits, such as mango, papaya and citrus fruits (Jacobi et al., 2001). Furthermore, it has been observed that heat treatments can affect a wide range of fruit ripening processes, such as control of ripening, fruit softening, pigment metabolism, volatile production, carbohydrate metabolism and disease development (Jacobi et al., 2001; Talcott et al., 2005; Zhang et al., 2009). These effects depend on the type of heat treatment applied and duration of fruit exposure. Heat tolerance of different fruits depends on species, genotype, stage of fruit maturity, type and severity of heat treatment applied, and whether postharvest conditioning treatments have been given before heat treatment (Jacobi et al., 2001).

Several studies report about the effect of heat treatments on an increase of heat shock proteins (HSPs), antioxidant enzymes and phytochemicals such as carotenoids and phenolic compounds. Ghasemnezhad et al. (2008) found an increase in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities after hot water treatments in mandarins. Talcott et al. (2005) found an increase in polyphenols and carotenoids and better antioxidant activity in hot water treated mangoes, compared with control. Moreover, Djioua et al. (2009) reported an effect of heat treatments on an increase in total carotenoids and vitamin C content in mango fruit. These results indicate that heat treatments prolong postharvest life of some fruits and promote an increase of bioactive compounds (Gonzalez-Aguilara et al., 2010).

5.1 Responses to heat treatment

Several authors suggest that the main response to thermal stress is the synthesis of plant hormones such as abscisic acid (ABA), salicylic acid (SA), sulphosalicylic acid (SSA) and MJ (Wahid et al., 2007), which modulate a numerous gene expressions.

Among secondary metabolites, carotenoids and some terpenoids are the main compounds enhanced by HTs which are involved in photo protection in the cell due to energy storage capacity. These molecules interact with membrane lipids providing thermo-stability of membrane and lowering susceptibility to lipid peroxidation under high temperatures (Penuelas and Munne-Bosch, 2005). Carotenoids also potentially play an important role in protecting the fruit tissue by scavenging ROS.

In addition, many studies have reported effects of UV-C and also heat treatments on an induction of some plant hormones, secondary metabolites and antioxidant enzymes (Figure 2.8 and 2.9) (Ghasemnezhad et al., 2008; Gonzalez-Aguilara et al., 2010). It has been found that HTs induce an increase in the activity of SOD, CAT and the ascorbate-glutathione cycle. It has been proposed recently that HSPs act synergistically with the above-mentioned enzymes or otherwise they might be related to the induction of these enzymes (Wahid et al., 2007). The production of these enzymes, in addition to the induction of carotenoids, is very important in maintaining and prolonging the postharvest life of fruits preventing oxidative damage in cell tissue acting as antioxidants. These antioxidants can help in controlling oxidative reactions caused by ROS and free radical species in living tissues and the inhibition of lipid peroxidation in foods caused by processing and during storage (Cisneros- Zevallos, 2003).

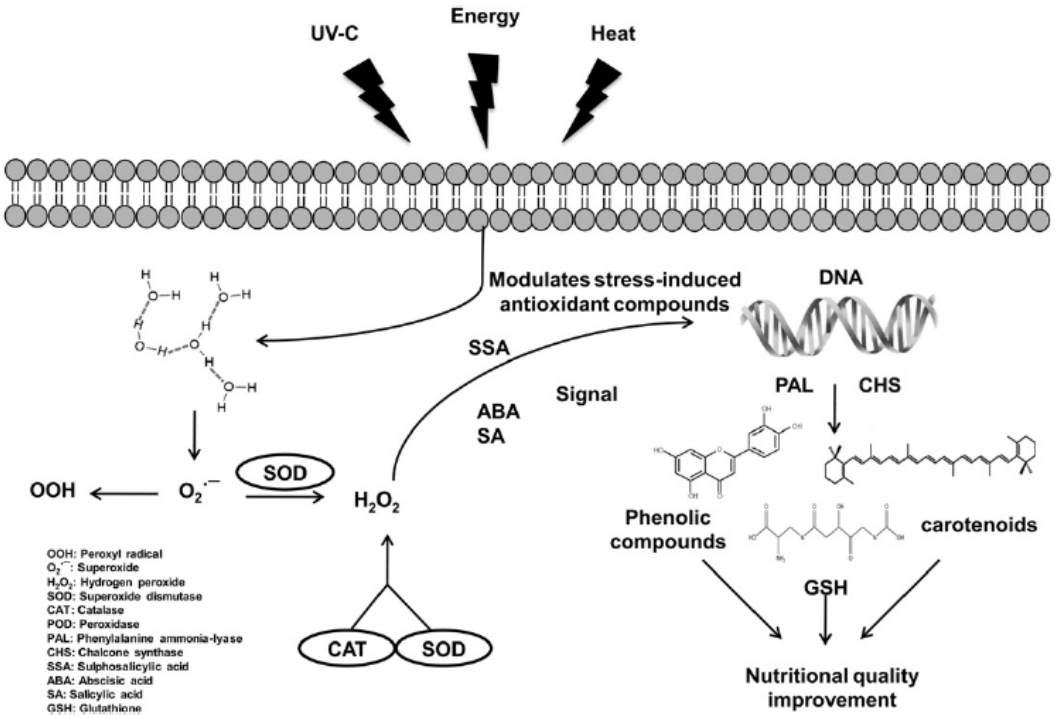


Figure 2.8 Hypothetical model representing possible induction of some plant hormones, secondary metabolites and antioxidant enzymes by UV-C and heat treatments (Gonzalez-Aguilara et al., 2010).

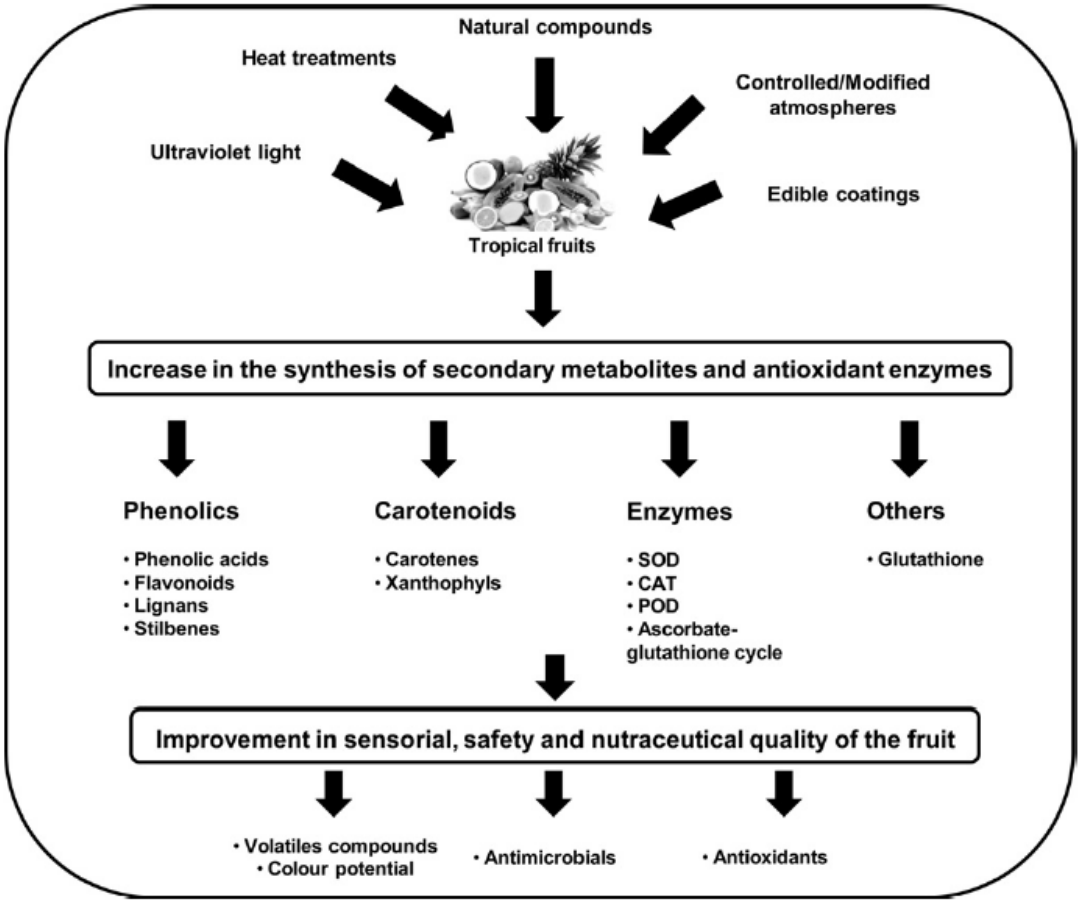


Figure 2.9 Controlled postharvest stress-type applied to tropical fruit which induces the synthesis of secondary metabolites and antioxidant enzyme system (Gonzalez-Aguilara et al., 2010).

5.2 Effects of heat treatment during storage at low temperature

There are many reports about the effects of heat treatment on physiological changes during low temperature storage. Klein and Lurie (1992) found that hot air treatment could alleviate storage disorders including chilling injury (CI). In cucumber fruit, hot air treatment at 36-40 °C for 24 h before storage at 5 °C reduced CI, respiratory rates and electrolyte leakage, compared with non-heated fruit (Hirose, 1985). It was suggested that hot water treatment induced the production of heat shock proteins (Klein and Lurie, 1991; Lafuente et al., 1991) and that these proteins conferred enhanced tolerance to chilling temperatures. Use of hot water treatment as a general postharvest treatment had become increasingly important due to its ease, non-toxic nature and variety of benefits (McCollum et al., 1995).

Lurie and Klein (1991) found that holding tomatoes at 36-40 °C for 3 days before storage at 2 °C for 3 weeks prevented CI and the fruit ripened normally when transferred to 21°C. Holding mangoes at 38 °C for 24 h or 48 h before storage at 5 °C inhibited the development of CI (McCollum et al., 1993). Heat treatments (hot air treatment) have to hold the product in air at 36-40 °C for hrs to days to be effective. However, on the commercial scale, treatment with hot air may be difficult to process. Therefore, an alternative method of heat water treatment has become more interested and more effective to induce chill tolerance in commercial operations than is hot air (McCollum et al., 1995). Lafuente et al. (1997) reported that hot water treatment over 50 °C for 1-3 mins could improve citrus fruit resistance to CI.

Surassawadee Promyou et al. (2008) found the effects of hot water treatment on the delaying of peel blackening during cold storage (4 °C) in ‘Hom Thong’ (cv. Gros Michel) banana. This effect was related to an increase in the ratio of unsaturated to saturated fatty acids, a decrease in LOX activity, reduced membrane degradation (MDA), reduced expression of a catechol oxidase gene and lower catechol oxidase activity. However, there was no clear change of free phenolics in the peel as a result of the hot water treatment.

5.3 Effects of heat treatment on extend postharvest life

Hot water treatment plus controlled atmosphere was an effective treatment combination to extend the postharvest life of mangoes, without adversely changing the nutritional profile of the fruit (Kim et al., 2007). They reported that gallic acid was identified as the major polyphenolic present in mangoes, followed by six hydrolysable tannins that constituted about 98% of the total polyphenolics indentified. It was found that gallic acid, total hydrolysable tannins, total soluble phenolics and antioxidant capacity significantly decreased throughout fruit ripening from mature-green to full ripe stages. In contrast, Talcott et al. (2005) reported an increase in

hydrolysable tannins in ‘Tommy Atkins’ mangoes during ripening. It indicated that the different growing condition or harvest years might affect on antioxidant compounds among fruits. However, hot water immersion was not a major factor influencing gallic acid, total hydrolysable tannins, total soluble phenolics and antioxidant capacity in mangoes during ripening (Talcott et al., 2005; Kim et al., 2007).

5.4 Effects of heat treatment on nucleic acid and protein metabolism

Heat treatment can also affect on nucleic acid and protein metabolism. For example, during application of a thermal stress, the expression of most housekeeping and ripening related genes decreases noticeably while the expression of genes corresponding to heat shock proteins (*HSPs*) increases (Vicente et al., 2006).

In hot air treated grape berries, the tolerance to subsequent lethal low temperatures were observed and also induced expression of *Hsp70* transcripts (Zhang et al., 2005). *HSPs* may induce ROS, followed by the production of oxygen radical scavengers such as SOD, POX and CAT (Vicente et al., 2006).

5.5 Effects of heat treatment on antioxidant system

According to antioxidant system, it was reported that heating ‘Fortune’ mandarins at 37 °C for 3 days induced 2.5-, 1.4- and 1.2-fold increases in the activities of CAT, SOD and APX, respectively. And that the differences in the activities produced by the heat treatment were maintained during cold storage (Sala and Lafuente, 1999). In addition, a combination of hot water (a rinse at 62 °C for 20s) and conditioning (pre-storage at 16 °C for 7 days) treatments synergistically reduced chilling injury development in grapefruit during cold storage at 2 °C, suggesting that the treatments may activate different chilling tolerance responses (Sapitnitskaya et al., 2006). In the case of strawberries, the treatment at 42 - 48 °C for 3 h in air delayed fruit ripening and reduced fungal attack (Vicente et al., 2002).

Hot air and UV-C treatment can increase levels of phenolics and ascorbic acid, enhancing PAL, SOD activities (involved in removing ROS) (Lemoine et al., 2010). Ghasemnezhad et al (2008) reported that hot water treatment could increase SOD, POD, and CAT activities in mandarins. Talcott et al. (2005) found the effects of hot water treatment on an increase of polyphenols and carotenoids and higher antioxidant capacity in mango. Djioua et al. (2009) reported an increase of total carotenoids and vitamin C content in mango fruit after hot water treatment.

5.6 Effects of heat treatment on membrane lipid

5.6.1 Lipid peroxidation of membrane

During oxidantive stress, ROSs can cause membrane damage by lipid peroxidation process. The basic reactions of lipid peroxidation are demomstrated in (Figure 2.10). Polyunsaturated fatty acids contain one or more methylene groups positioned between *cis* double bonds. The methylene groups are highly reactive to oxidizing agents and their hydrogen atoms are removed to form carbon-centered radicals, as shown in compound 1 (Figure 2.10) (Marnett, 1999).

Lipid peroxidation generates a complex variety of products, many of which are reactive electrophiles. Many carbonyl compounds including hexanal, 4-hydroxynonenal, and other saturated and a,b-unsaturated aldehydes and ketones can react with protein and DNA and are toxic and mutagenic. MDA appears to be the most mutagenic product of lipid peroxidation whereas 4-hydroxynonenal is the most toxic (Marnett, 1999).

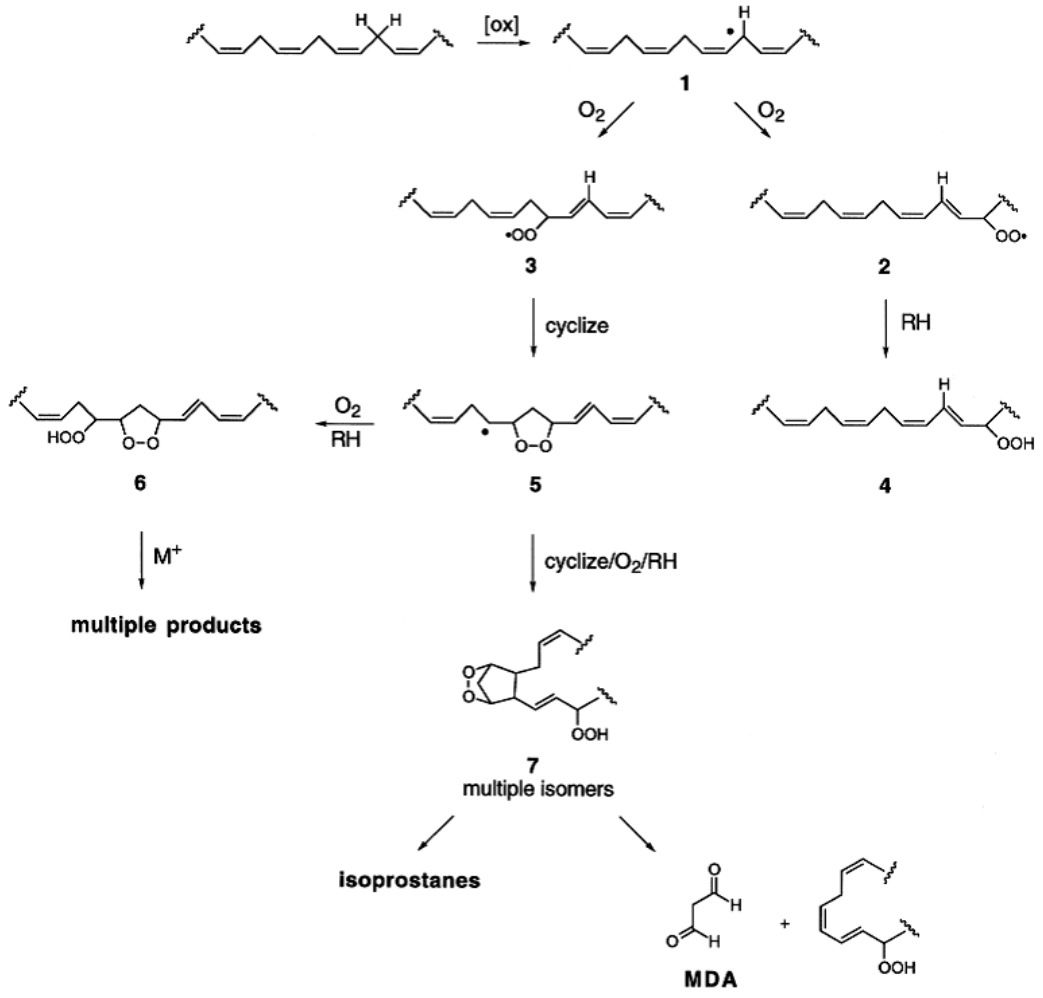


Figure 2.10 Pathways of lipid peroxidation (Marnett, 1999).

Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation, as biomarker for oxidative stress (Nielsen et al., 1997). It can react with thiobarbituric acid (TBA) to form an intensely colored chromogen (Marnett, 1999).

The red MDA:TBA condensation product is a 1:2 adduct with a TBA , at each end of the molecule (Figure 2.11). The formation of MDA-derived pigments usually requires low pH and elevated temperatures (80-100 °C) (Janero, 1990).

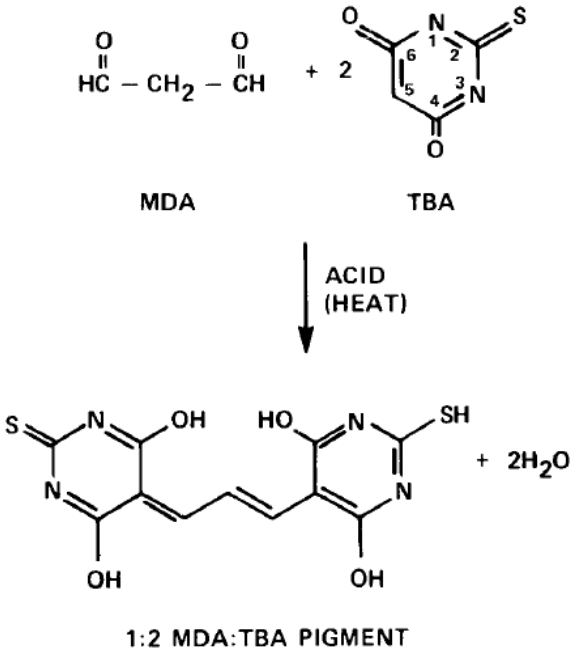


Figure 2.11 Formation of the fluorescent red 1:2 adduct between MDA and TBA (Janero, 1990).

5.6.2 Effect of heat treatment on electrolyte leakage

The membrane is responsible for the selective inflow and outflow of molecules, ions, and water. Stresses, such as temperature abuse, radiation and toxic chemicals can induce loss of normal physiological processes, membrane leakage, and tissue injury (Fan and Sakora, 2005). Electrolyte leakage may be a useful tool to predict an ability of vegetable to tolerate irradiation since electrolyte is generally considered as an indirect measure of plant cell membrane damage (Fan and Sakora, 2005). Increased membrane permeability and increased rates of ion leakage are associated with chilling of sensitive tissue (Saltveit, 2002). In pomegranate fruit, the development of chilling injury was highly correlated to the skin browning and EC leakage (Mirdehghan et al., 2007).

Heat treatment slowed not only the rate of ripening-related fruit softening, but also ripening-related membrane changes, such as leakage, microviscosity and the increase in fatty acid saturation during low temperature storage (Klein and Lurie, 1990). Thus, a pre-storage heat treatment affected fruit plasma membrane so as to increase fruit store ability and slow the after-storage ripening. In apple fruit, for instance, it was found that the loss of unsaturated fatty acids during ripening occur more rapidly in control fruits, although the fatty acid content in apples from both treatments was similar after storage (Lurie et al., 1995). Heat treatment may activate fruits to store better by an initial acceleration of some processes of ripening, which then recover during the subsequent storage period (Lurie et al., 1995).

Storage at chilling temperature (2.5 °C) resulted in a significant increase in electrolyte leakage; hot water immersion could reduce this effect (McCollum et al., 1995). Hot water immersion has been shown to inhibit CI in citrus fruit, which exhibited the reduction of electrolyte leakage. However, electrolyte leakage averaged increased 4.5-6.0% in cucumber fruit immediately after treatment with no significant difference among the treatments (McCollum et al., 1995).

Fruits held at 38 °C for 4 days prior to storage at 0 °C showed increase of membrane microviscosity and sterol content, phospholipid fatty acid became more saturated and there was increased electrolyte leakage from apple fruit disc. Then, microviscosity and leakage were lower in heated fruit, and increased more slowly during ripening at 20 C than in control apples (Lurie et al., 1995).

Hot water treatment at 38 °C or 42 °C for 30 min resulted in no visible injury to the fruit and no increase in electrolyte leakage, one indicator of thermal injury. Immersion treatment also caused a transient increase in respiration and ethylene production, but these effects had subsided by 48 h after treatment. The most pronounced effect of hot water treatment was an inhibition of ACC oxidase activity (McCollum et al., 1995).

The increase in polyamine levels could be a defense mechanism against stresses, involving protection of cell membrane lipids, and could be responsible for lower electrolyte leakage. Vicente et al. (2006) suggested that heat treatment could induce acclimation of pomegranates to low temperature and in turn reduce chilling injury, possibly by a mechanism involving an increase in both polyamine levels and the unsaturated/saturated fatty acid ratio. Mirdehghan et al. (2007) found that heat treatment by hot water dip at 45°C could increase polyamine levesl and maintain higher of the unsaturated/saturated fatty acid ratio during storage, which was highly correlated with the lower in electrolyte leakage. This result suggested that heat treatment could maintain more membrane integrity and fluidity. As a result, the severity of chilling injury symptoms was reduced in heat-treated fruit.

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

1. Plant materials

Hands of 'Hom Thong' banana (*Musa acuminata*, AAA group) fruits obtained from a local farm in Saraburee province, were used for hot water treatment. The maturity of banana was about 80% (90 days after flowering). For *CAT* and *SOD* cloning, 'Blue field' bananas (Musa acuminata, AAA group) were harvested from a commercial plantation in Hilo, Hawaii.

2. Instruments

2.1 Equipments for hot water treatment

- Digital water bath (DAIHAN LABTECH CO., LTD)

- Thermometer

2.2 Equipments for packaging and storage

- Cartoon box, size 34 x 26 x 12 cm

- Phytotron room

2.3 Equipments for measuring some physiological changes and sample collecting

- Colorimeter (Color Reader CR-10, KONICA MINOLTA SENSING, INC., Japan)

- Penetrometer

- Knife and cutting board

- Liquid nitrogen

- Aluminium foil

- Deep freezer -80 °C

2.4 Equipments for antioxidants extraction and analysis

- Mortars and pestles
- Spatula
- Nalgene tube, 50 ml
- Refrigerated centrifuge (Universal 32R, Hettich, Germany)
- Spectrophotometer (Agilent Technology, USA)

2.5 Equipments for H_2O_2 staining

- Blade
- Slide
- Microscope (BX51, Olympus, Japan)
- Dichroic mirror units (DM500, U-MWB2)
- Fluorescence generator (BH2-RFL-T3, Olympus, Japan)
- Digital camera unit (DP70, Olympus, Japan)

2.6 Equipments for RNA extraction

- Deep freezer -80°C
- Mortars and pestles
- Spatula
- Refrigerated centrifuge (Universal 32R, Hettich, Germany)
- Eppendorf tubes, 1.5 ml
- Spectrophotometer (Agilent Technology, USA)
- Vortex mixture

2.7 Equipments for electrophoresis

- Horizontal gel electrophoresis (MiniRun GE-100, Hangzhou BIOER Technology Co., Ltd., China)

- Gel DocTM 2000 and UV transilluminator (Bio-Rad, USA)

- Microwave oven

2.8 Equipments for real time PCR

- Individual PCR tube ^{TM} Low tube strip, WHT (Bio-Rad, USA)

- PCR tube strips, Flat Cap Strips (Bio-Rad, USA)

- CFX96^{TM} Real- time System (Bio-Rad, USA)

3. Chemicals and reagents

3.1 Chemicals for MDA analysis

- 5% and 15% (w/v) trichloroacetic acid (TCA)

- 0.5% thiobarbituric acid (TBA)

3.2 Chemicals for H_2O_2 content analysis

- Phosphate buffer (pH 6.5)

- Hydroxylamine

- Titanium sulphate

- Sulfuric acid

3.3 Chemical for H_2O_2 staining

- 10 μM H_2DCFDA

3.4 Chemicals for carotenoid content analysis

- 80% acetone

3.5 Chemicals for ascorbic acid content analysis

- 2% Dinitrophenylhydradzine (DNPH) in 4.5 M sulfuric acid

- 6% metaphosphoric acid in 2 M acetic acid

- 2% 2, 6-dichlorophenolindolphenol (DCIP)

- 2% thiourea in 5% metaphosphoric acid

- 90% sulfuric acid

- Standard ascorbic acid

3.6 Chemicals for glutathione content analysis

- Potassium phosphate buffer (pH 7.0)

- 5, 5-dithiobis-2-nitrobenzoic acid (DTNB)

- NADPH

- Glutathione reductase (60 units/ml)

- Standard glutathione (reduced form, GSH)

3.7 Chemicals for phenolic compound analysis

- 80% EtOH

- 4 N NaOH

- 6 N HCl

- Ethyl acetate

- Distilled water

- Folin-Ciocalteu's phenol reagent (Fluka, Switzerland)

- Standard gallic acid

3.8 Chemicals for flavonoid content analysisi

- 5% $NaNO_2$

- 10% $AlCl_3$

- 1 M NaOH

- (+)-catechin

3.9 Chemicals for total antioxidant activity

- 0.2 mM of DPPH (1, 1-diphenyl-2-picrylhydrazyl) ethanolic solution

- 80% EtOH

3.10 Chemicals for RNA extraction

- Liquid nitrogen
- Plant RNA Purificaion Reagent (Invitrogen, USA)
- Phenol: Chloroform: Isoamyl alchohol (25:24:1) (v/v)
- Chloroform: Isoamyl alcohol (24:1) (v/v)
- Absolute ethanol
- TE buffer (see in Appendix A)
- Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich Co., USA)
- Hydrogen peroxide
- 80% ethanol
- Sodium acetate (CH_3COONa) (Sigma-Aldrich Co., USA)
- Sodium chloride (NaCl)
- DNaseI (Takara Bio Inc., Japan)

3.11 Chemicals for electrophoresis

- Agarose (Research Organics, USA)
- TBE buffer (Tris Borate EDTA) (see in Appendix A)
- Ethidium bromide (Gibco BRL, USA)
- RNA loading dye (see in Appendix A)
- DNA marker (100 bp DNA ladder, BioLabs Inc.)

3.12 Chemicals for real-time PCR

- M-MLV reverse transcriptase (Promega, USA)
- 2X Prime Q- Master Mix (GENET BIO, Korea)

2. METHODS

2.1 Hot water treatment and measurement of fruit quality parameters

2.1.1 Hot water treatment

Hands of 80% mature green, as a commercial stage of harvest, 'Hom Thong' bananas (*Musa acuminata*, AAA group) were obtained from a local farm in Saraburi Province. Hands of banana were immersed in hot water at 50 °C for 10 minutes (the most effective treatment from previous experiment) (Jintana Chancharoenrit, 2002). For control treatment, bananas were immersed in water at room temperature (26 – 27 °C) for 10 minutes. Then, they were kept in the carton boxes, 4 replicates (1 hand/replication) for each treatment. Afterward, the bananas were stored at 25 °C until ripening (Experiment 1). To study the responses of hot water treated banana at low temperature during a commercial transportation, bananas were stored at 14 °C for 8 days before storage at 25 °C for 8 days for Experiment 2. Physiological changes were determined on day 0 (after treatment), 2, 4, 6, 8 and 10 for Experiment 1 and on day 0 (after treatment), 8, 10, 12, 14 and 16 for Experiment 2. For gene expression, the samples were also analyzed after 0, 1 and 3 hr after treatment.

2.1.2 Measurement of some physiological changes

2.1.2.1 Peel color change

Banana peel color was determined by measuring parameters L value, and hue value with a colorimeter (Color Reader CR-10, KONICA MINOLTA SENSING, INC., Japan). Color values of each fruit were computed as means of three measurements taken from blossom end, middle and stem end of the fruit peel. L value and hue value of each treatment were compared.

2.1.2.2 Pulp firmness

Pulp firmness was measured by using penetrometer at the same regions of the fruits as color measurement. Firmness values were multiplied by 9.807 to Newton (N) unit (Jintana Chancharoenrit, 2002).

2.2 Determination of lipid peroxidation

Banana peels were collected on day 0, 2, 4, 6, 8, and 10 for Experiment 1 and on day 0, 8, 10, 12, 14 and 16 for Experiment 2 and then stored at -80 °C until the analysis of lipid peroxidation. In order to investigate membrane damage, lipid peroxidation was measured by estimating the concentration of malondialdehyde (MDA), using thiobarbituric acid-reactive substances (TBARS) assay according to Zhang et al. (2005) with some modifications.

Banana peel (1.5 g) was homogenized with 10 ml of 5% (w/v) trichloroacetic acid (TCA). After centrifugation, 3 ml of 0.5% thiobarbituric acid (TBA) in 15% TCA was added to 5 ml of supernatant. Then, the mixer was boiled for 20 min in water bath (80°C), quickly cooled and centrifuged at 6,000 g for 15 min. The supernatant was used to measure the absorbance at 450, 532 and 600 nm.

The MDA concentration (μmol/g FW) was calculated according to the equation: $6.45x\ (A_{532} - A_{600}) - 0.56xA_{450}$ (Zhang et al., 2005).

2.3 Determination of electrolyte leakage

Banana peels were collected on day 0, 2, 4, 6, 8, and 10 for Experiment 1 and on day 0, 8, 10, 12, 14 and 16 for Experiment 2. Then, fresh banana peels (5.0 g) were cut into small pieces with the 0.7 mm diameter cork border, and incubated at room temperature with distilled water. During incubation, samples were agitated using a shaker at a speed of 100 min^{-1}. Electrotrical conductivity of the bathing solution was measured at 1 min (C_1) and 60 min (C_{60}) of incubation using a conductivity meter. The samples were then autoclaved (121°C) for 20 min, and total conductivity (C_T) was then measured after cooling. Electrolyte leakage (E) was calculated from the following equation: $E = (C_{60}-C_1)/C_Tx100$ (Fan and Sokorai, 2005).

2.4 Determination of H_2O_2 content

Banana peels were collected on day 0, 2, 4, 6, 8, and 10 for Experiment 1 and on day 0, 8, 10, 12, 14 and 16 for Experiment 2 and then stored at -80°C until the analysis of H_2O_2 contents. For H_2O_2 staining, fresh banana peels were used.

2.4.1 H_2O_2 concentration

H_2O_2 was extracted by homogenized peel tissue (1.0 g) with 8 ml phosphate buffer (pH 6.5) containing 1 mM hydroxylamine, at 0 °C. After centrifuge at 6,000 g for 25 min, 1ml of 0.2% titanium sulphate in 20% H_2SO_4 (v/v) (prepare before use) was added to 3 ml supernatant and then centrifuged at 6,000 g for 15 min. To determine H_2O_2 content, the supernatant was measured at 410 nm as described by Jana and Choudhuri (1982). According to standard curve, H_2O_2 content was shown as μmol/g FW.

2.4.2 H_2O_2 staining

H_2O_2 accumulation was visualized according to the modified method of Zhao et al. (2009). The banana peel sections were incubated in 10 μM H_2DCFDA for 10 min at room temperature. Green fluorescence in the section was detected using a microscope (BX51, Olympus, Japan) equipped with dichroic mirror units (DM500, U-MWB2) and fluorescence generator (BH2-RFL-T3, Olympus). Images were photographed using a digital camera unit (DP70, Olympus).

2.5 Determination of non-enzymatic antioxidants

In order to analyze the content of antioxidants, including ascorbic acid (AA) (vitamin C), carotenoid, glutathione, free and bound polyphenolics, free and bound flavonoids and total antioxidant activity, banana peels were collected on day 0, 4, 6, 8, and 10 for Experiment 1 and on day 0, 8, 12, 14 and 16 for Experiment 2 and then stored at - 80°C until analysis.

2.5.1 Carotenoids

Carotenoids were extracted from peel tissue (1.0 g) with 8ml of 80% acetone. Then, the extract was incubated at 4 °C for 24 h. After centrifuge at 6,000 g for 5 min, the absorbance of the supernatant was determined at 480, 645, 663 and 710 nm. The carotenoid concentration was calculated from the following equation:

$$(A_{480} + 0.114(A_{663} - A_{710}) - 0.638(A_{645} - A_{710})) * 8 * 1000)/112.5 * FW$$

The carotenoid contents were expressed as μmol/g FW (Kirk and Allen, 1965).

2.5.2 Ascorbic acid (AA)

Total AA content was determined using the dinitrophenylhydradzine (DNPH) method with some modifications followed by Shin et al. (2007). Peel tissue samples (1.0 g) were extracted with 10 ml of 6% metaphosphoric acid in 2 M acetic acid. The extracted was filtered through #1 Whatman filter paper. After that, 2 ml of supernatant and 0.1 ml of 2% 2, 6-dichlorophenolindolphenol (DCIP) were mixed and incubated at room temperature for 1 h. Then, 2 ml of 2% thiourea in 5% metaphosphoric acid and 1 ml of 2% dinitrophenylhydrazine (DNPH) in 4.5 M sulfuric acid were added, incubated at 60 °C for 3 h. To stop the reaction, the tubes were placed in an ice bath and 5 ml of 90% sulfuric acid (on ice) were slowly added. Total AA was quantified by measurement of the absorbance at 540 nm and compared to the standard curve. The concentration was expressed as ascorbic acid on a fresh weight basis, mg g-1.

2.5.3 Glutathione

Glutathione including reduced glutathione (GSH) and oxidized glutathione (GSSG) were assayed using the sensitive and specific enzyme method as described by Castillo and Greppin (1988). Peel tissue samples (1.5 g) were homogenized with 5 ml of cold potassium phosphate buffer (60 mM KH_2PO_4, pH 7.0), containing 0.1 M KCl and 2.5 mM EDTA. After centrifuge, the supernatant (200 µl) were added to the reaction mixture for each measurement.

To determine GSH, the reaction mixture consisted of 2 ml of 60 mM K-phosphate buffer containing 2.5 mM EDTA (pH 7.5) and 0.66 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB). After incubation for 10 min, the mixtures were measured the absorbance at 412 nm to determine the concentration of reduced DTNB (which is proportional to GSH). The concentrations were calculated according to standard curves of GSH.

For determination of total glutathione (GSH and GSSG), the supernatant was added to the reaction mixer, containing 2 ml of 60 mM K-phosphate buffer containing 2.5 mM EDTA (pH 7.5) and 0.66 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 20 µl of 20 mM NADPH and 20 µl of 60 units/ml glutathione reductase. After incubation for 10 min, the mixtures were measured the absorbance at 412 nm to determine the concentration of reduced DTNB (which is proportional to GSH or GSSG).

The concentrations of total glutathione were calculated according to standard curve of GSH. Then, the GSSG concentrations were calculated from subtraction of total glutathione with GSH concentrations.

2.5.4 Free and bound phenolics

Free and bound extracts of banana were preceded according to the method of Choi et al. (2006) with some modifications. Banana peel (2.0 g) was ground with liquid nitrogen to fine powder, and then 10 ml of 80% ethanol were added. The homogenate were vortexed for 1 min and centrifuged at 9,000 rpm for 5 min at 25 °C. Then, the supernatants were collected for free extract analysis.

The residues from the free compound extraction was hydrolyzed with 2 ml of 4 N NaOH for 1 h and adjusted to pH 2 with 625 µl of 6 N HCl. After centrifuged for 5 min, the supernatant was collected and the aqueous phase was extracted 2 times with 2 ml of ethyl acetate. The organic extracts were incubated at 40 °C to dryness and re-dissolved with 200 µl of 80% ethanol and then added up with distilled water to 1.5 ml. Both free and bound extracts were then stored at -20 °C until using for the analysis of phenolics, flavonoids and antioxidant activity.

Phenolic contents in banana peel were determined according to the Folin-Ciocalteu colorimeter method with some modifications (Choi et al., 2006). For the free phenolic analysis, 50 µl of extract was mixed with 25 ul of a 50% Folin-Ciocalteu's phenol reagent and 500 ul of 2% $NaCO_3$ solution. For the bound phenolics, 100 µl of extract was used for a reaction. After incubation at room temperature for 30 min, the absorbance at 750 nm was measured. Phenolic contents were expressed as mg gallic acid equivalents per 1 g of banana peel.

2.5.5 Free and bound flavonoids

Flavonoid contents in banana peel were determined by a colorimeter method with some modifications (Choi et al., 2006). For the free flavonoid measurements, 63 µl of free extract was mixed with 687 µl of distilled water and 38 µl of 5% $NaNO_2$ solution. After 5 min, 75 µl of 10% $AlCl_3$ was added. After 6 min, 250 µl of 1 M NaOH and 138 µl of distilled water were added to the reaction. For the bound flavonoid analysis, 200 µl of bound extract and 550 µl of distilled water were used for the reaction without adding 138 µl of distilled water at the last steps. The reaction was mixed well and the absorbance at 510 nm was measured. The flavonoid contents were expressed as mg (+)-catechin equivalents per 1 g of banana peel.

2.5.6 Antioxidant activity

The antioxidant activity in banana peels was analyzed by the determination of DPPH radical scavenging activity effect on DPPH radical according

to the method of Choie et al. (2006) with some modifications. For the determination in free extract, 100 µl of free extract and 300 µl of 80% ethanol were mixed with 400 µl of 0.2 mM DPPH ethanolic solution. For the bound extract analysis, 400 µl of bound extract was mixed with 400 µl of 0.2 mM DPPH ethanolic solution. The reaction was mixed well and incubated for 10 min in the dark. The absorbance of sample was measured with spectrophotometer at 520 nm against a blank of ethanol without DPPH. The DPPH radical scavenging activity (%) was calculated by the following equation:

$$\text{Radical scavenging (\%)} = (1 - A_{sample}/A_{control}) * 100$$

Where A_{sample} is the absorbance in the presence of extract and $A_{control}$ is the absorbance in the absence of extract.

2.6 Gene expression

Total RNA was extracted by using Plant RNA purification reagent (Invitrogen, USA). Banana peel from 4 replications of each treatment was pooled and then freeze dried. Freeze dried sample (0.1 g) was extracted by using 0.8 ml extraction buffer. After incubated for 5 min at room temperature, the sample was centrifuged at 12,000 rpm for 10 min, and then the supernatant was collected. After that, 0.2 ml of 5 M NaCl was added to the supernatant, followed by 0.6 ml of chloroform: isoamyl (24:1), mixed well and centrifuged at 14,000 rpm for 10 min (4 °C). Supernatant was collected and equal volume of isopropyl alcohol was added. After 30 min of incubation at -20 °C, the RNA pellet was precipitated by centrifugation at 14,000 rpm for 10 min (4 °C). RNA pellet was washed with 1 ml of 80% EtOH, let it dry at room temperature and dissolved with 30 ul of TE. DNase-treated RNA (2 µg) was used for cDNA synthesis by M-MLV reverse transcriptase (Promega, USA). Quantitative real-time PCR was performed by using 2x Prime Q-master mix with CFX96 Real-time system (Bio-Rad). Gene specific primers were designed based on NCBI reference to conserved nucleotide sequences of *SOD* and *CAT* genes (Table 5.1). The PCR conditions were (1.) 95.0 °C for 10:00 (2.) 95.0 °C for 0:30 (3.) 62.0 °C for 0:30 (4.) 72.0 °C for 0:30 + Plate Read (5.) Go to step 2, 39 more times (6.) 95.0 °C for 0:10 (7.) Melt curve 65.0 to 95.0, increment 0.5 °C for 0:05 + Plate Read and (8.) End. Gene expressions were investigated after 0, 1, and 3 hr after treatment, and on day 0, 2, 4, 6, 8, and 10 for *Experiment 1* and on day 0, 8, 10, 12, 14 and 16 for *Experiment 2*. Triplicate quantitative PCR experiments will be performed for each sample and the expression values obtained were normalized against *EF-1α*.

Table 3.1 Primers for Real-time PCR .

Primer name	Sequence	Tm (°C)	Product size
EF1-F	GGGGGATTGACAAGCGTGTTATCG	58.15	116
EF1-R	ACCACGTTCACGCTCAGCCT	58.65	
CAT-F	GCGCATACCGGAGCGCGTCGT	64.16	165
CAT-R	GCTGCCACGCTCGTGGATGAC	60.69	
SOD-F	GGTGGAGGTCATATCAACCACTCG	56.84	164
SOD-R	CTGCAAAGCAGCACCTTCTGC	57.18	

2.7 Data analysis

Four replicates per treatment were done for all measurements. The means were compared by the independent sample *t-test* at a significant level of 0.05 ($P < 0.05$) using SPSS software version 14. The data were shown as means ± S.E. (standard error).

CHAPTER IV

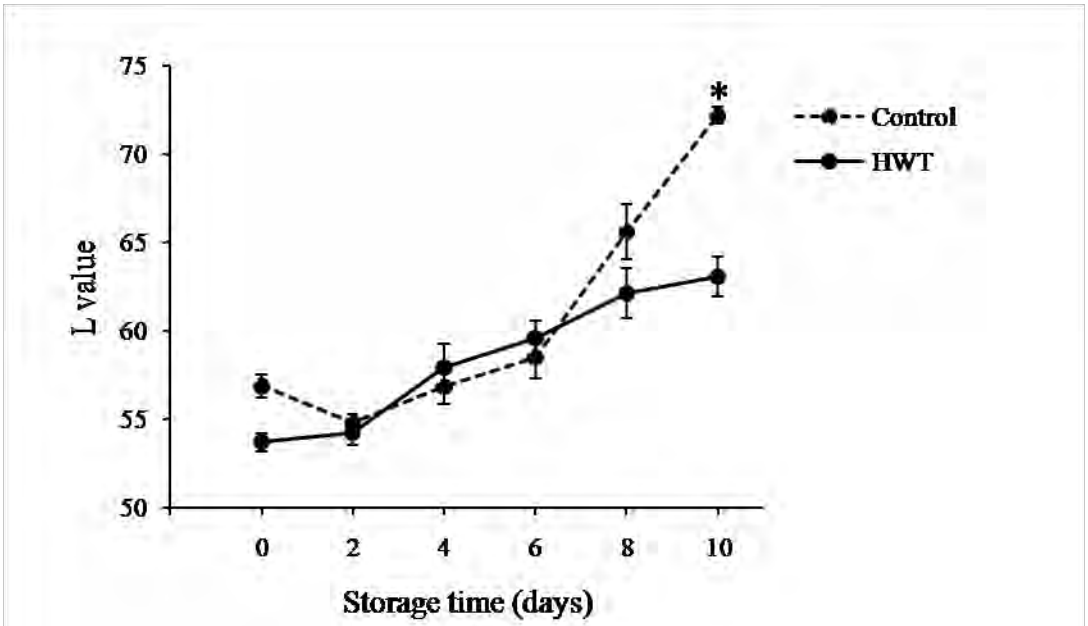
RESULTS AND DISCUSSION

1. RESULTS

1.1 Effects of hot water treatment on fruit quality parameters and antioxidant contents during storage at 25 °C

1.1.1 Lightness (L value)

As the banana fruit began to ripen, the L value increased and was similar in hot water treated and in control fruits. The L value increased very sharply in control fruit from day 6 to day 10 but rose only slightly in hot water treated fruit. There was a significant difference in the L value on day 10 (Figure 4.1).

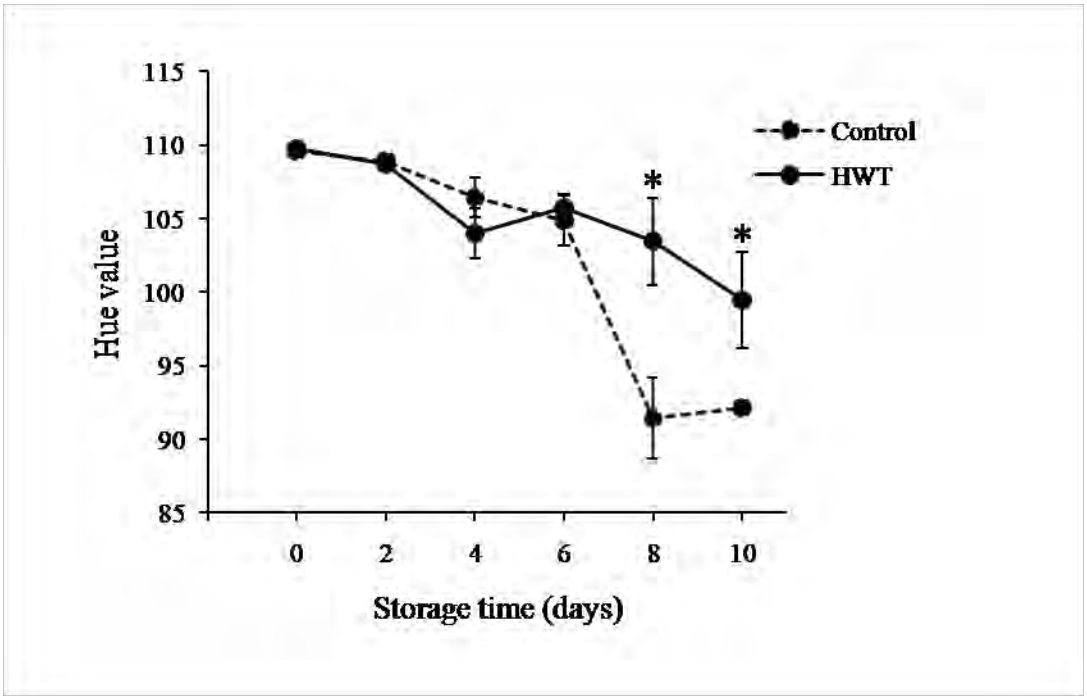


* indicates the significant difference between control and hot water treatment (HWT) ($P < 0.05$).

Figure 4.1 L value of banana peel during storage at 25 °C.

1.1.2 Hue value

Hue values (hue angle) decreased during ripening, indicating a change from green to yellow. Hot water treated banana fruits showed higher hue value than those of control. The significant differences were found on day 8 and day 10, indicating the delay ripening of banana fruits in the hot water treatment (Figure 4.2).

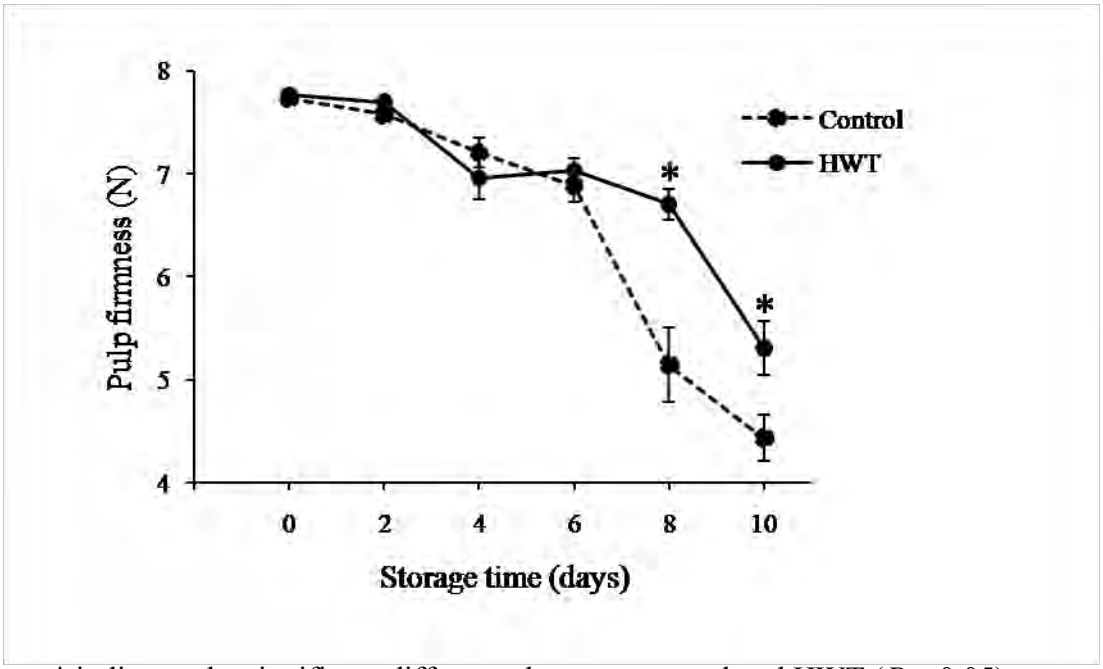


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.2 Hue value of banana peel during storage at 25 °C.

1.1.3 Pulp firmness

Pulp firmness decreased during banana ripening. Higher pulp firmness was found in hot water treated banana fruits on day 8 and 10 of storage. Therefore, hot water treatment can delay the pulp softening. Significant differences were shown on day 8 and 10 (Figure 4.3). Changes of hue value were correlated with that of pulp firmness (r = 0.966, $P < 0.01$).



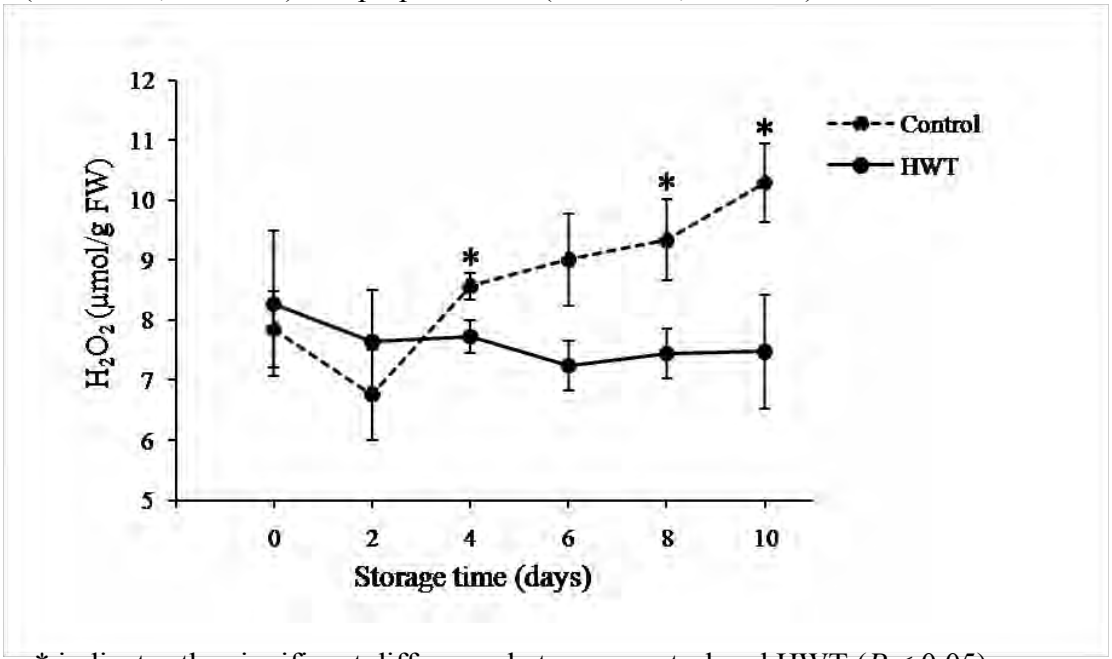
* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.3 Pulp firmness of banana during storage at 25 °C.

1.1.4 H_2O_2 contents

1.1.4.1 H_2O_2 concentration

The H_2O_2 concentration in control banana tended to increase during ripening, while in hot water treated fruit, they were relatively stable. The H_2O_2 contents in hot water treatment were lower than those of control from day 4 to day 10. The significant differences can be found on day 4, day 8 and day 10 (Figure 4.4). In control fruit, increases of H_2O_2 content were negatively correlated with changes of hue value ($r = -0.828$, $P < 0.05$) and pulp firmness ($r = -0.863$, $P < 0.05$).



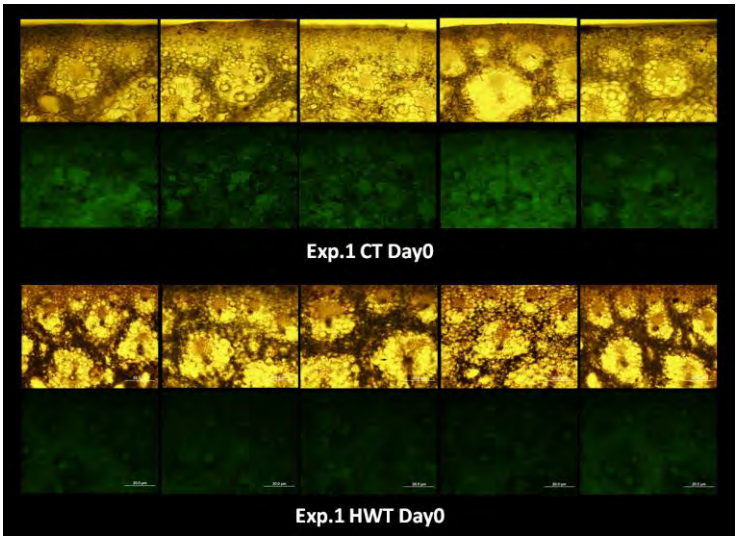
* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.4 H_2O_2 contents of banana during storage at 25 °C.

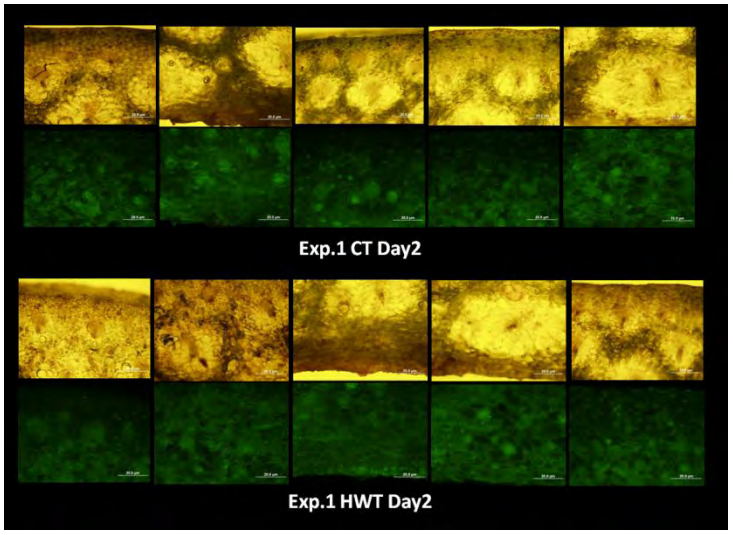
1.1.4.2 H_2O_2 staining

The accumulation of H_2O_2 in peel tissue was determined by staining the tissue with fluorescent dyes that indicate ROSs within the cell. The cells that showed the highest fluorescence are the vascular cells (Figure 4.5 and 4.6). A stronger fluorescence, which indicates higher H_2O_2 content, was shown in hot water treated fruits on day 6 and day 8 (Figure 4.6). The fluorescence tended to be weaker when banana fruits began to ripen in both control and hot water treated fruits. However, the density of fluorescence had no correlation with the H_2O_2 accumulation determined by spectrophotometric assay, which showed an increase of H_2O_2 concentration in control fruit during storage period.

(A)



(B)



(C)

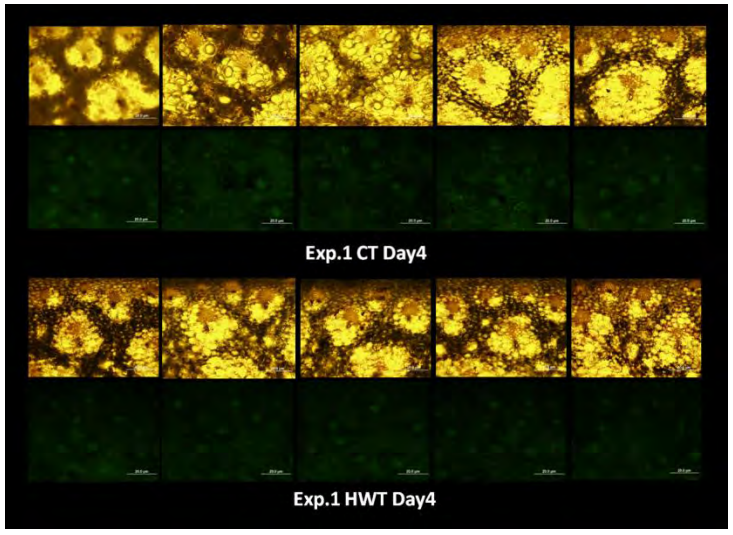
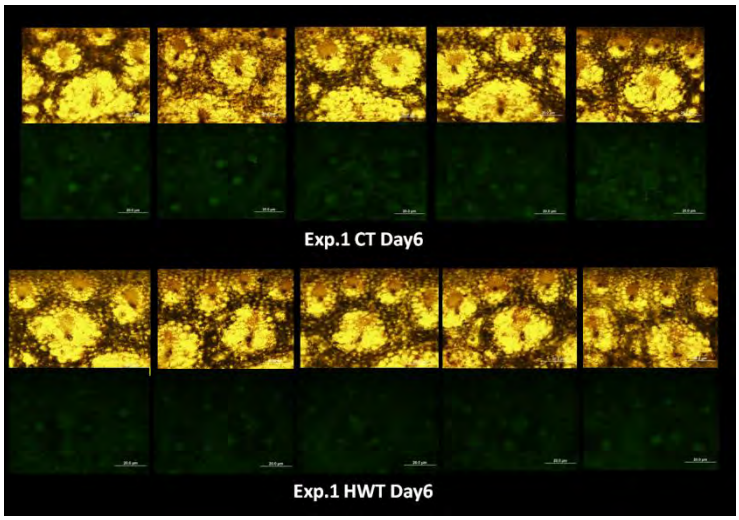
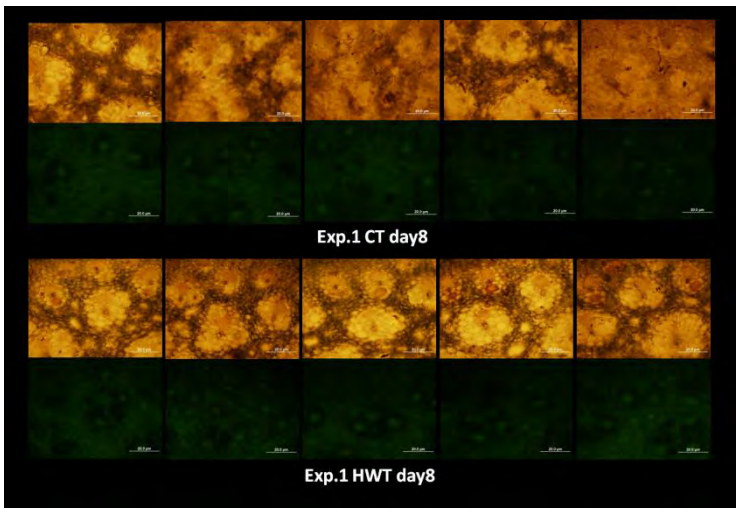


Figure 4.5 H_2O_2 staining of banana peel during storage at 25 °C. The upper rows of each treatment show normal peel tissue under light microscope: the lower rows show stained tissue with H_2DCFDA. (A) – (C) showed the H_2O_2 staining for day 0, 2, and 4, respectively.

(D)



(E)



(F)

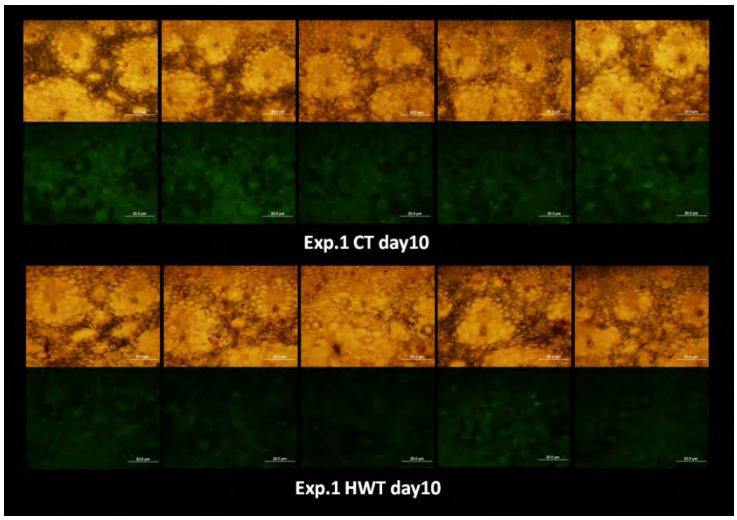
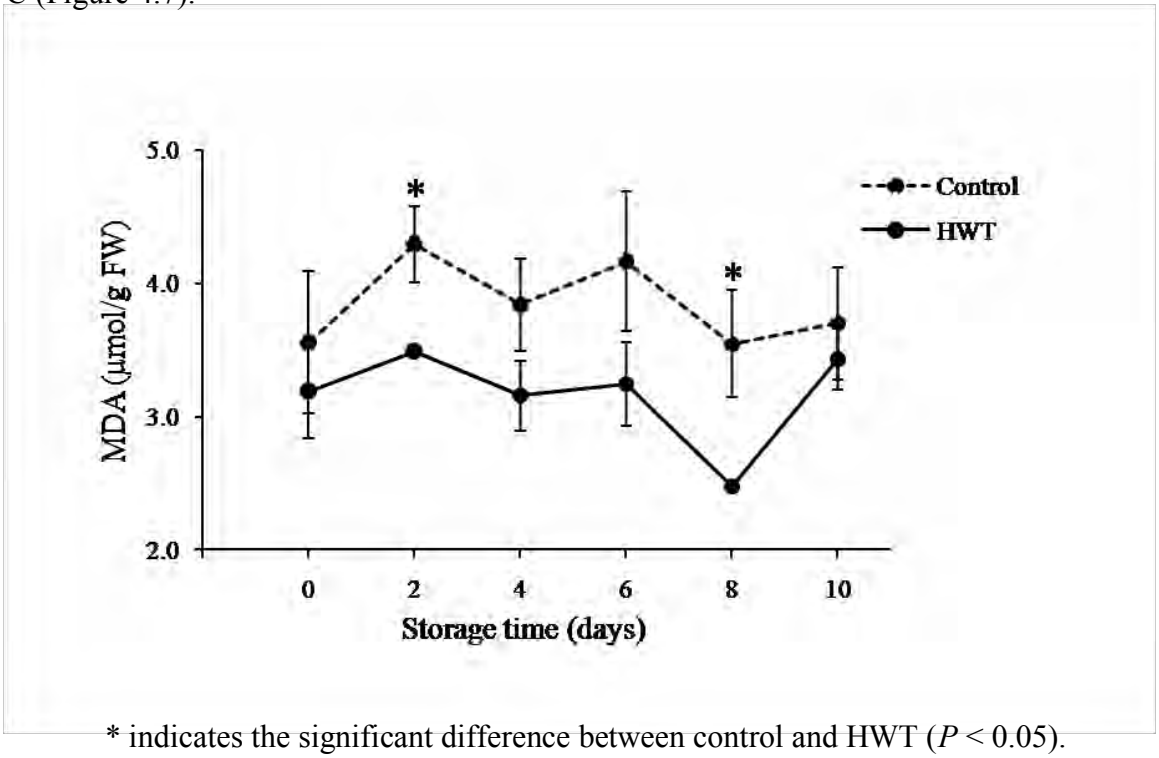


Figure 4.6 H_2O_2 staining of banana peel during storage at 25 °C. The upper rows of each treatment show normal peel tissue under light microscope: the lower rows show stained tissue with H_2DCFDA. (E) – (F) showed the H_2O_2 staining for day 6, 8, and 10, respectively.

1.1.5 MDA concentration

The patterns of MDA contents during storage slightly fluctuated slightly over time. However, hot water treated banana showed lower MDA contents than those of control. Significant differences were found on day 2 and 8, indicating a lower lipid peroxidation or membrane damage from hot water treated banana during storage at 25°C (Figure 4.7).

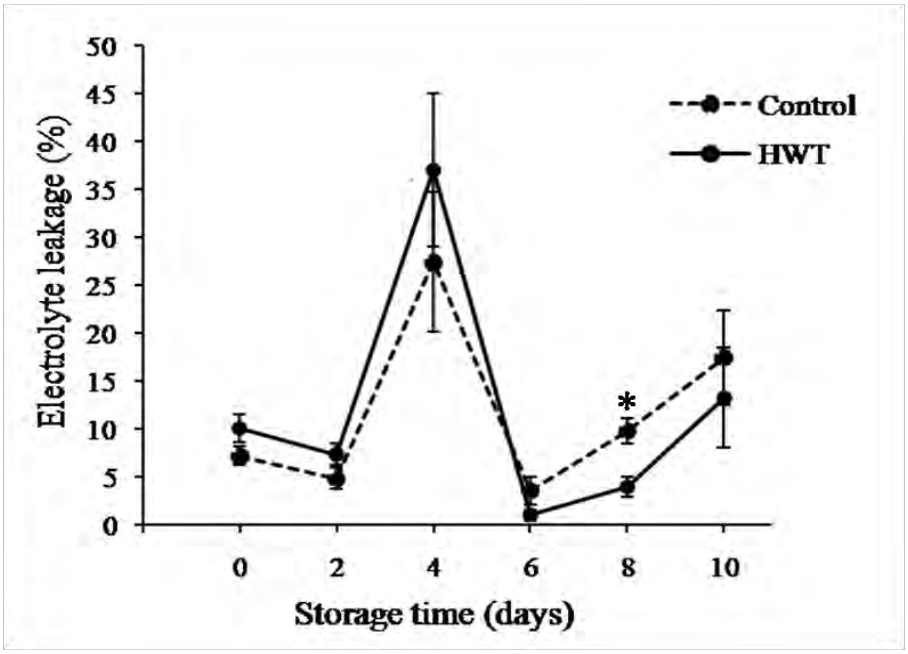


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.7 MDA contents of banana during storage at 25 °C.

1.1.6 Electrolyte leakage

In general, electrolyte leakage of banana fruit increased after storage for 4 days. Later the fruits exhibited a decrease in leakage on day 6 and which then, increased continuously until day 10. Higher electrolyte leakage could be found in hot water treated banana on day 0 to day 4. On day 6, 8, and 10, hot water treated banana exhibited lower electrolyte leakage than those of control fruit. The significant differences were found on day 8 (Figure 4.8).

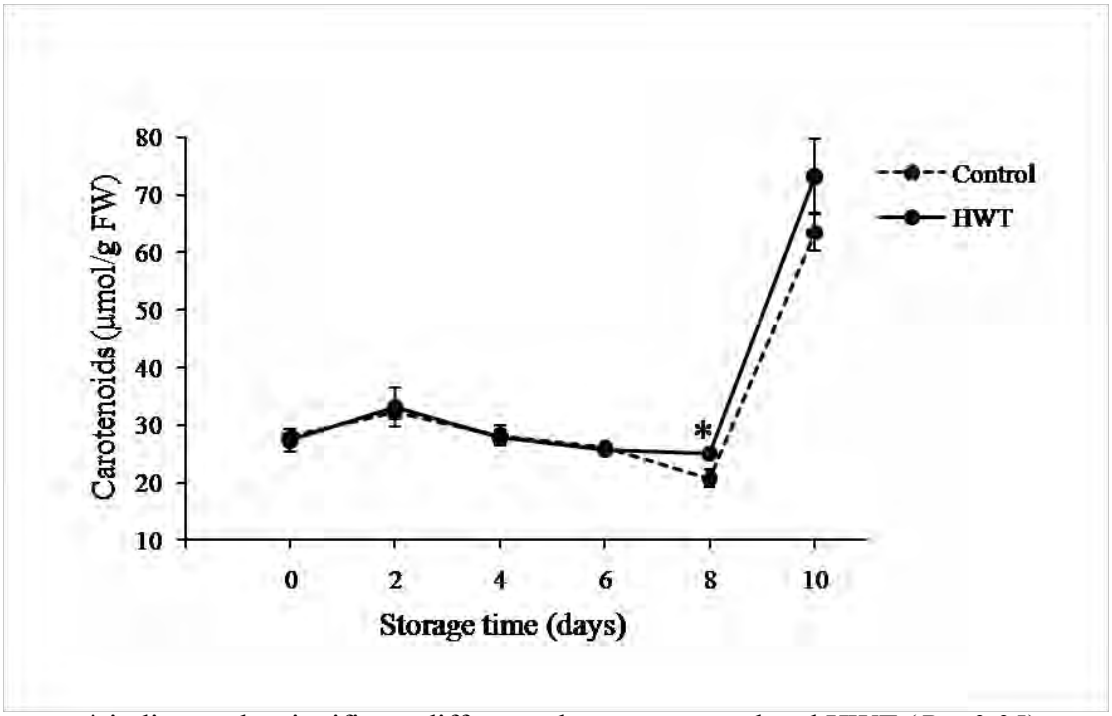


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.8 Electrolyte leakage of banana peel during storage at 25 °C.

1.1.7 Carotenoid concentration

According to hue value that indicated the delay in ripening of hot water treated banana, chlorophyll a and chlorophyll b content in hot water treatment were higher than that of control, significantly on day 8 and 10 (data not shown). While chlorophyll a and b decreased, carotenoid contents increased from day 8 to day 10. A significant difference was found on day 8 where hot water treated banana had higher carotenoid contents (Figure 4.9). In hot water treated fruit, carotenoid contents were negatively correlated with pulp firmness ($r = -0.821$, $P < 0.05$).

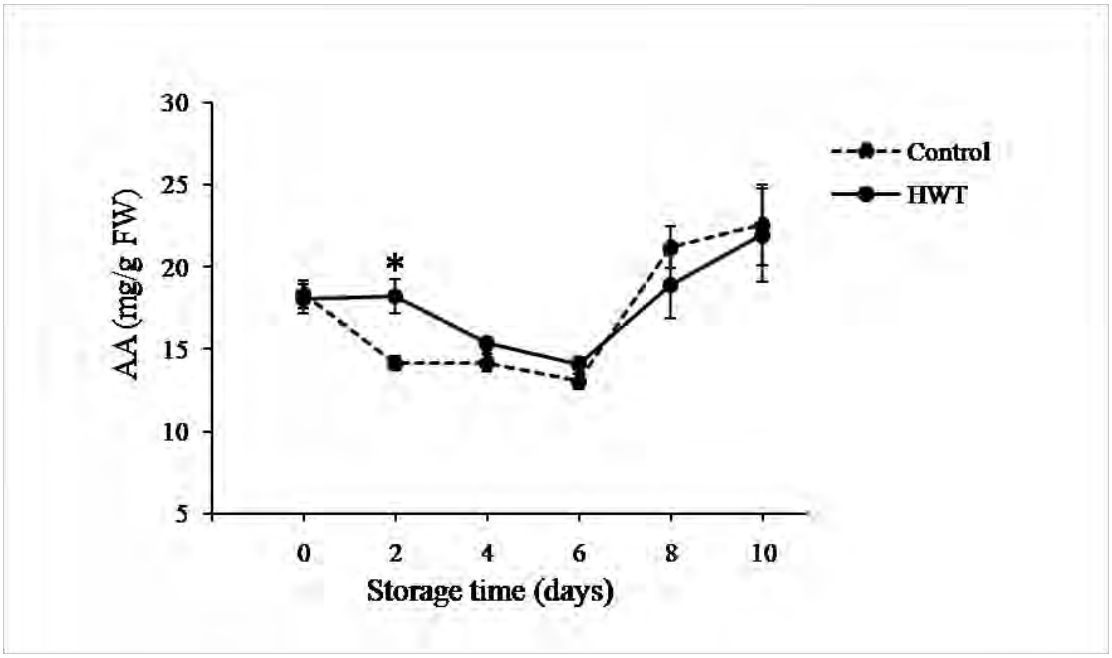


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.9 Carotenoid contents of banana peel during storage at 25 °C.

1.1.8 Ascorbic acid concentration

The ascorbic acid (AA) content of banana in control and hot water treatments decreased to a lower level than those of day 0 on day 2 to day 6. After which, they increased during ripening on day 8 and day 10. Higher AA contents in hot water treatment were found on day 2, 4 and 6. Later, on day 8 and day10, hot water treated bananas showed lower AA contents than those of control. The significant difference was shown on day 2 (Figure 4.10).

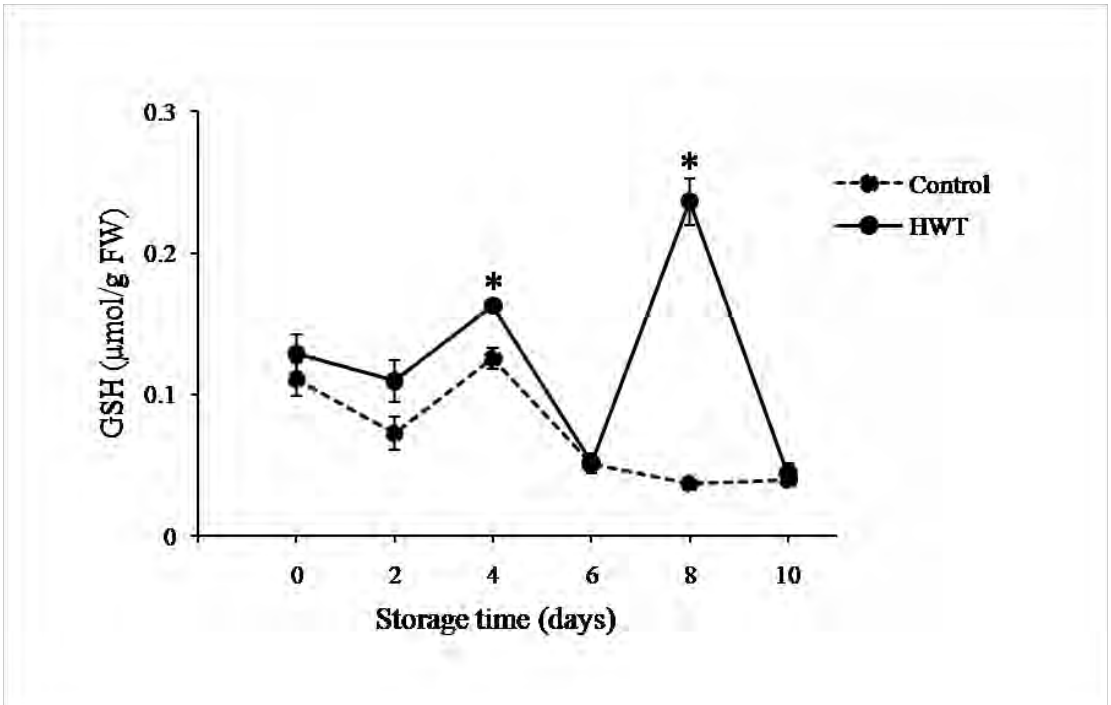


* indicates the significant difference between control and HWT($P < 0.05$).

Figure 4.10 Ascorbate contents of banana peel during storage at 25 °C.

1.1.9 GSH concentration

GSH contents in control and hot water treated banana slightly fluctuated from day 0 to day 6 and then increased sharply in hot water treated fruit on day 8 while decreased in control fruit. The higher GSH content in hot water treatment was significantly different from those of control on day 4 and day 8 (Figure 4.11). In hot water treated fruit, GSH contents were negatively correlated with MDA ($r = -0.835$, $P < 0.05$) contents.

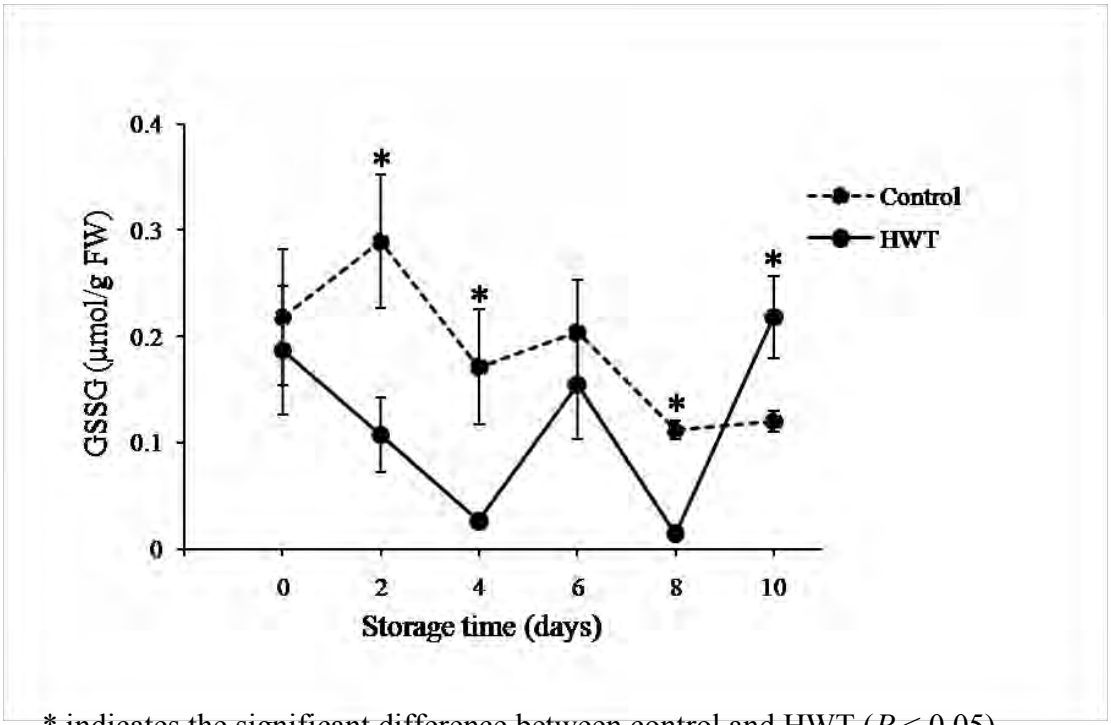


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.11 GSH contents of banana peel during storage at 25 °C.

1.1.10 GSSG concentration

GSSG content in hot water treated fruit tended to be lower than that of control from day 0 to day 8. The significant differences were found on day 2, 4 and 8. On day 10, GSH contents in hot water treatment increased significantly higher than in control fruit (Figure 4.12). In hot water treated fruit, GSSG contents were negative correlated with GSH contents (r = - 0.838, $P < 0.05$). In control fruit, GSSG contents were correlated with hue value (r = 0.865, $P < 0.05$), pulp firmness (r = 0.842, $P < 0.05$) and negatively correlated with H_2O_2 contents (r = -0.914, $P < 0.05$).

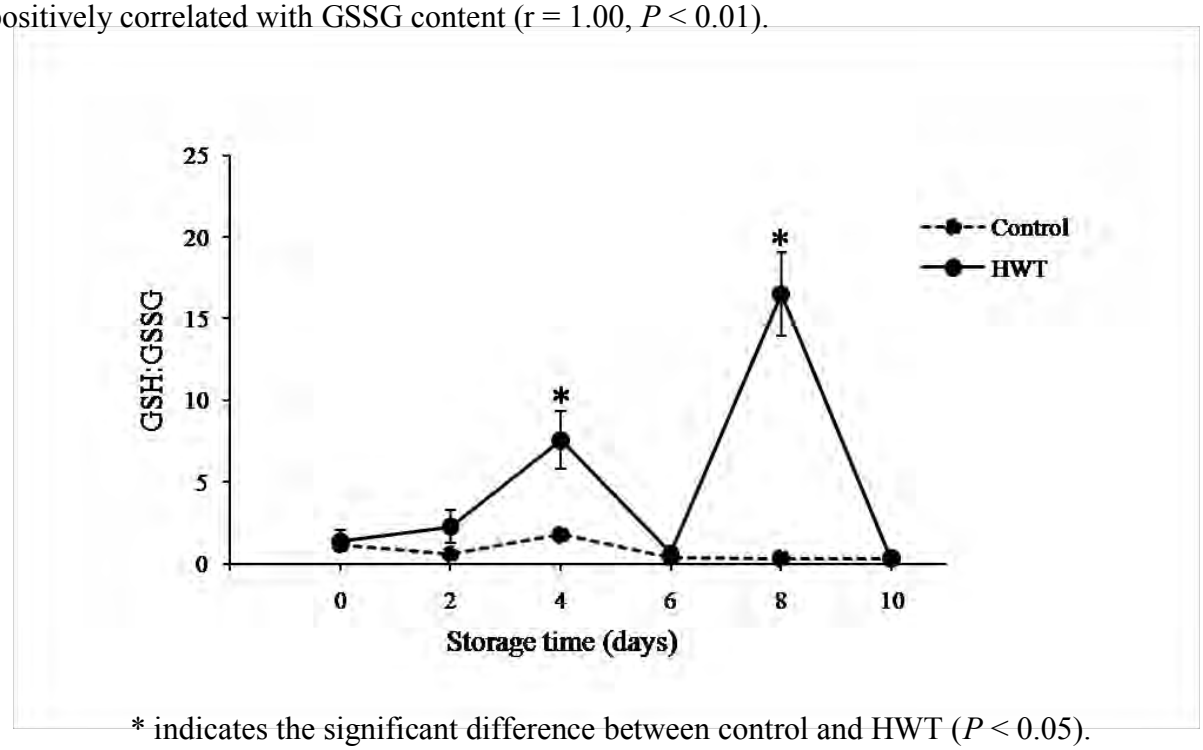


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.12 GSSG contents of banana peel during storage at 25 °C.

1.1.11 GSH/GSSG ratio

The ratio of GSH to GSSG (GSH/GSSG) was stable from day 0 to day 6. Then, on day 8, hot water treated banana fruit showed an increase in the GSH/GSSG ratio, while the control fruit GSH/GSSG ratio remained constant. The significant differences were found on day 4 and day 8. However, during fruit ripening on day 10, the ratio of GSH to GSSG decreased to same level as the initial storage (Figure 4.13). In control fruit, GSH/GSSG ratio was correlated with hue value ($r = 0.865$, $P < 0.05$), pulp firmness ($r = 0.842$, $P < 0.05$) and GSSG contents ($r = 1.00$, $P < 0.05$), but negatively correlated with H_2O_2 content ($r = -0.914$, $P < 0.05$). In hot water treated fruit, GSH/GSSG ratio was negatively correlated with GSH ($r = -0.838$, $P < 0.05$), but positively correlated with GSSG content ($r = 1.00$, $P < 0.01$).

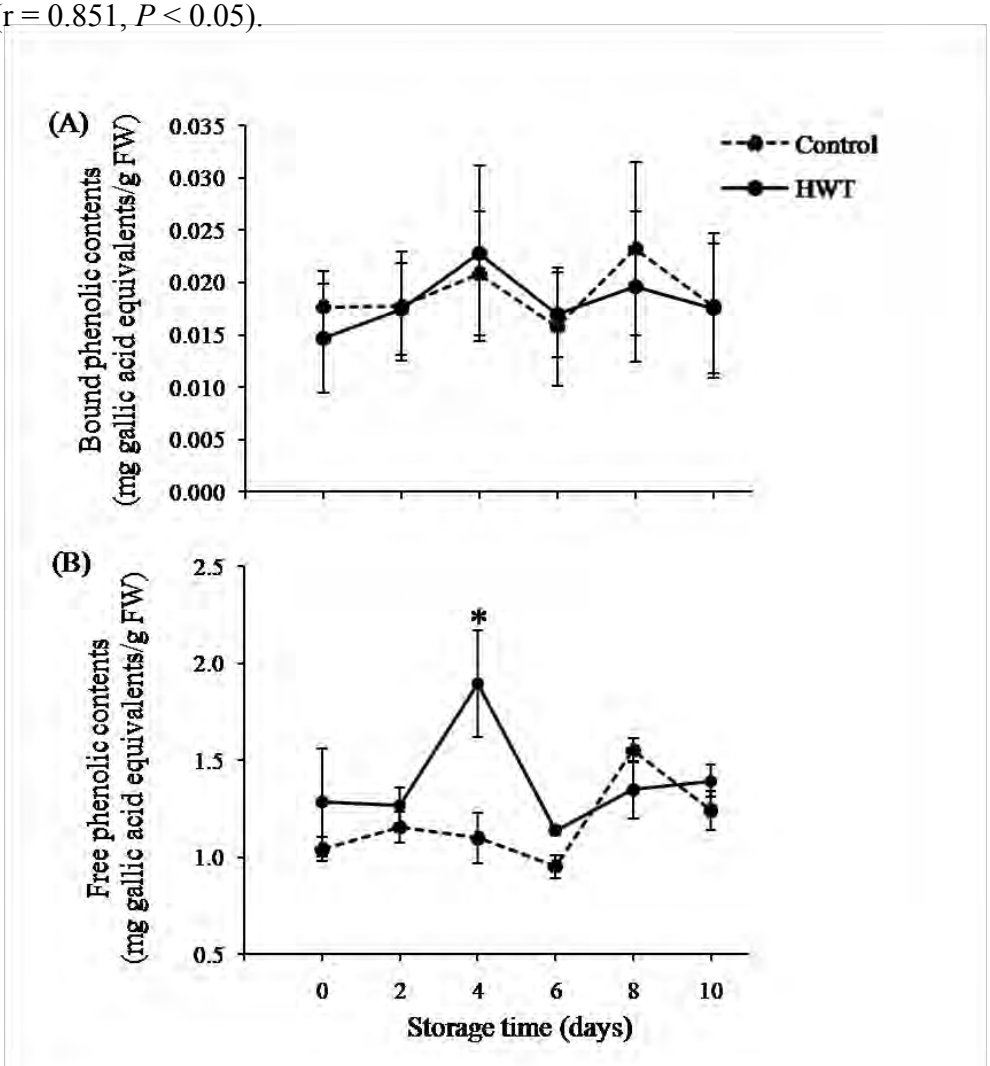


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.13 The ratio of GSH to GSSG (GSH/GSSG) contents of banana peel during storage at 25 °C.

1.1.12 Bound and free phenolic compound concentration

The bound phenolics of both control and hot water treated banana fruit increased from day 0 to day 4, and then fluctuated until day 10. The bound phenolics of hot water treated fruit tended to be lower than those of control fruit during ripening on day 8 and day 10. There were no significant differences among the treatments (Figure 4.14A). The free phenolics of hot water treated fruit were higher than those of control fruit on day 0, 2, 4, 6 and 10. The highest value of free phenolics was found on day 4 in hot water treated fruit. The significant differences were found on day 4 and day 6. However, there was no significant difference during fruit ripening on day 8 to day 10 (Figure 4.14B). In hot water treated fruit, free phenolic contents were correlated with electrolyte leakage ($r = 0.969$, $P < 0.05$) and bound phenolic contents ($r = 0.851$, $P < 0.05$).

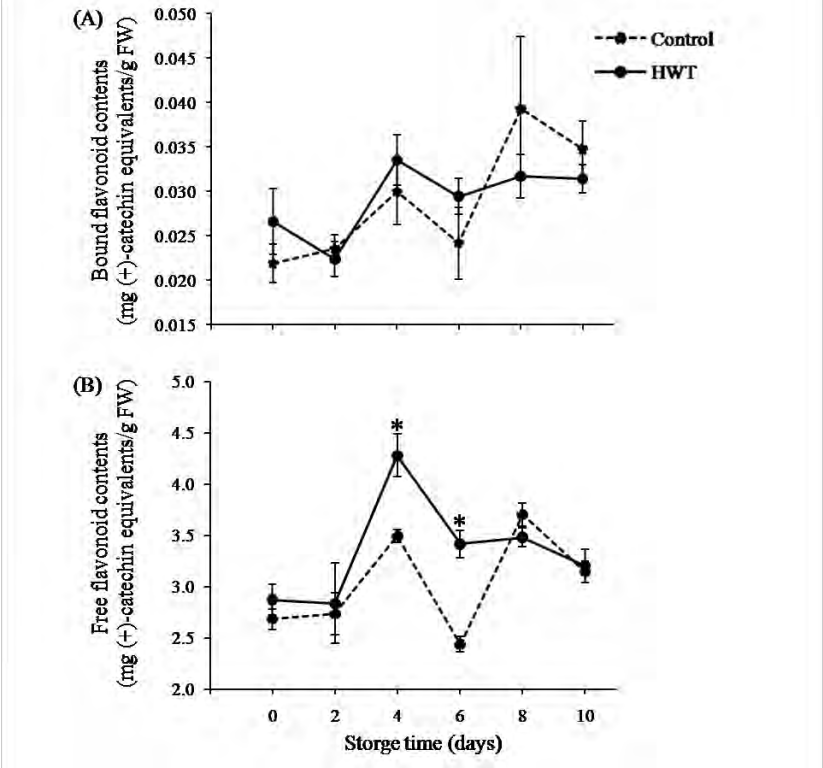


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.14 Bound (A) and free (B) phenolic contents of banana peel during storage at 25 °C.

1.1.13 Bound and free flavonoid concentration

The bound flavonoids of banana fruits were expressed as mg of (+)-catechin equivalents per 1 g of sample. The flavonoids of the banana fruits tended to increase during storage until ripening. Both hot water treated fruit and the control demonstrated similar trends in bound flavonoids before fruit ripening (day 4 and day 6) and during fruit ripening on day 8 and day 10. There was no significant difference between the treatments throughout the storage (Figure 4.15A). In control fruit, bound flavonoid contents were negatively correlated with hue value ($r = -0.923$, $P < 0.01$), pulp firmness ($r = -0.873$, $P < 0.05$), GSH ($r = -0.869$, $P < 0.05$), and GSH/GSSG ratio ($r = -0.860$, $P < 0.05$), but positively correlated with free phenolics ($r = 0.866$, $P < 0.05$). The free flavonoids of hot water treated fruit tended to be higher than those of control from day 0 to day 6. The significant differences were found on day 4 and day 6. However, when fruits began to ripen (day 8 to day 10), there was no significant difference among the treatments (Figure 4.15B). In control fruit, free flavonoid contents were correlated with bound phenolics ($r = 0.941$, $P < 0.01$) and bound flavonoids ($r = 0.863$, $P < 0.05$). In hot water treated fruit, free flavonoid contents were also correlated with bound phenolics ($r = 0.916$, $P < 0.05$) and bound flavonoids ($r = 0.825$, $P < 0.05$).

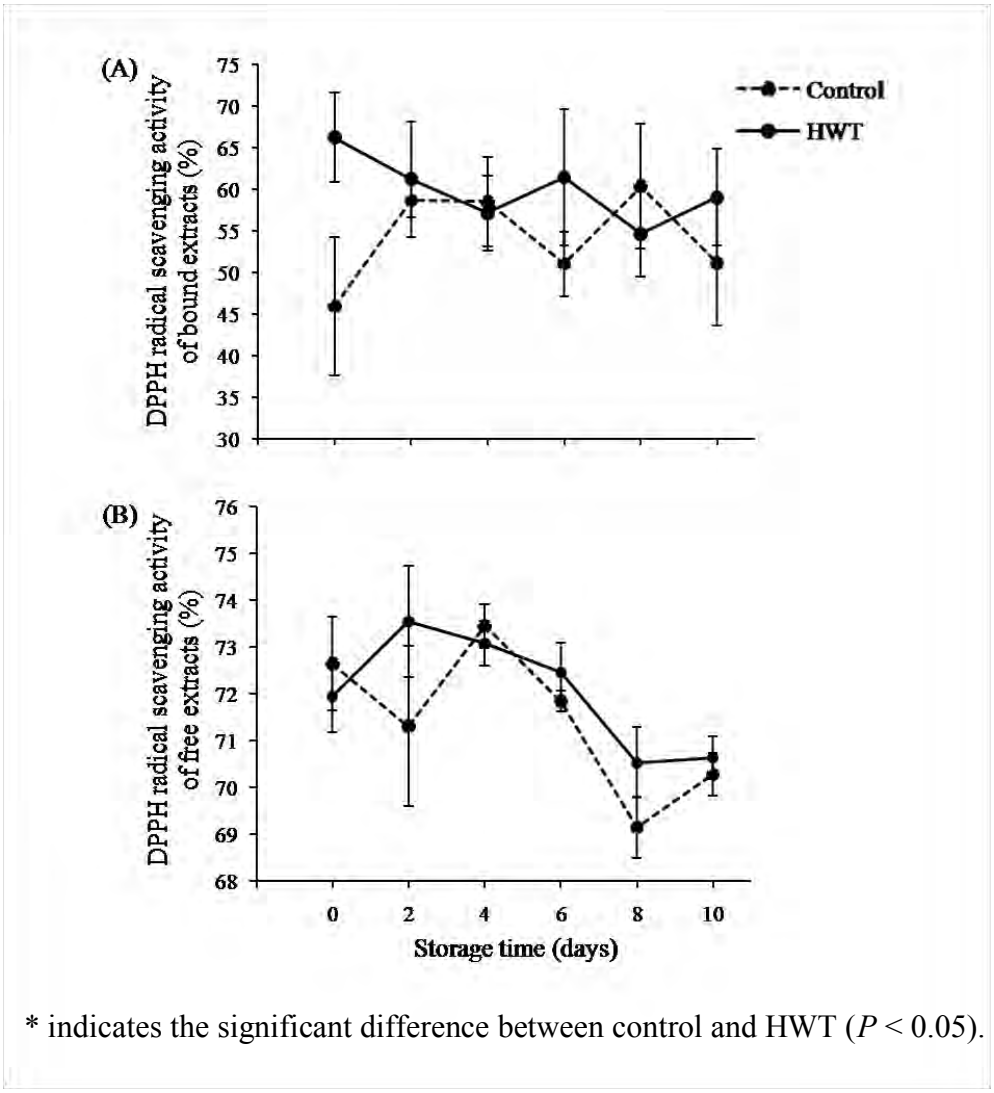


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.15 Bound (A) and free (B) flavonoid contents of banana peel during storage at 25 °C.

1.1.14 DPPH radical scavenging activity in bound and free extracts

The DPPH radical scavenging activity, which indicates antioxidant activity, was determined in both bound and free extracts. For the antioxidant activities of bound extracts, hot water treated banana fruits tended to have higher activity than those of control, except on day 4 and day 8. In general, there were slight fluctuations of antioxidant activity of bound extracts in both control and hot water treatments. The significant difference could not be found throughout storage (Figure 4.16A). In free extracts, the antioxidant activity tended to decrease during storage. The hot water treated fruits showed higher antioxidant activity; however, there was no significant difference (Figure 4.16B). In control fruit, DPPH radical scavenging activities were correlated with hue value (r = 0.834, $P < 0.05$), GSH (r = 0.894, $P < 0.05$), but negatively correlated with free phenolics (r = -0.817, $P < 0.05$).



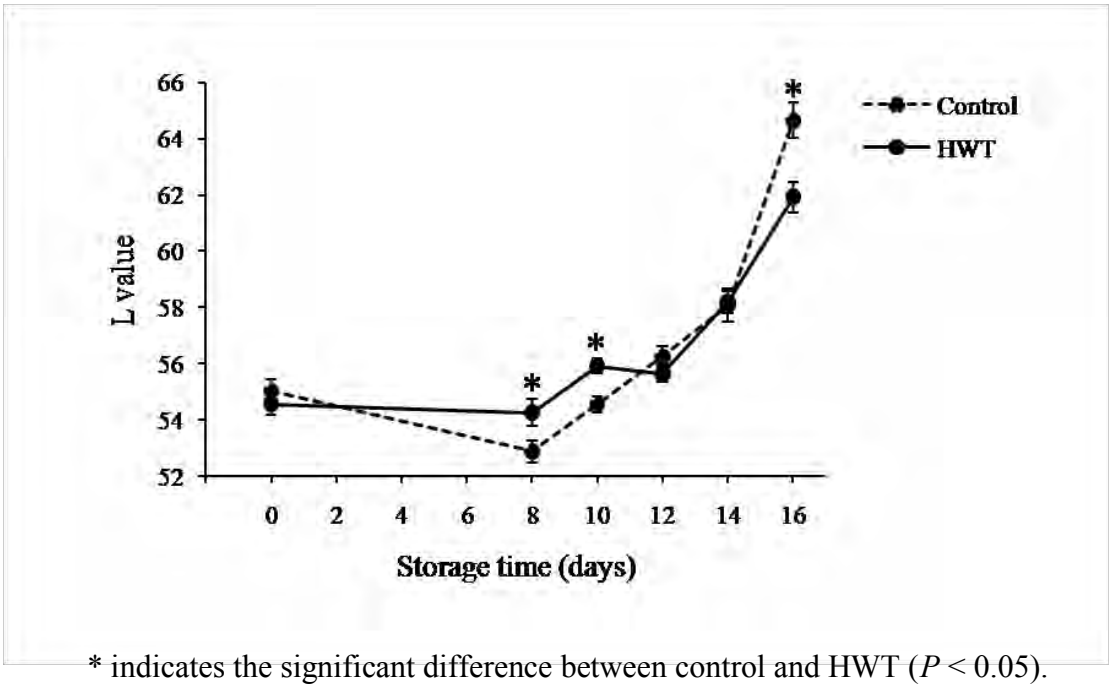
* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.16 DPPH radical scavenging activity in bound (A) and free (B) extracts of banana peel during storage at 25 °C.

1.2 Effects of hot water treatment on fruit quality parameters and antioxidant contents during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.1 L value

The L value increased during banana fruit ripening. Hot water treated banana fruit had a significantly higher L value on day 8 and 10. However, on day 16, L value in hot water treatment was significantly lower than those of control fruit (Figure 4.17).

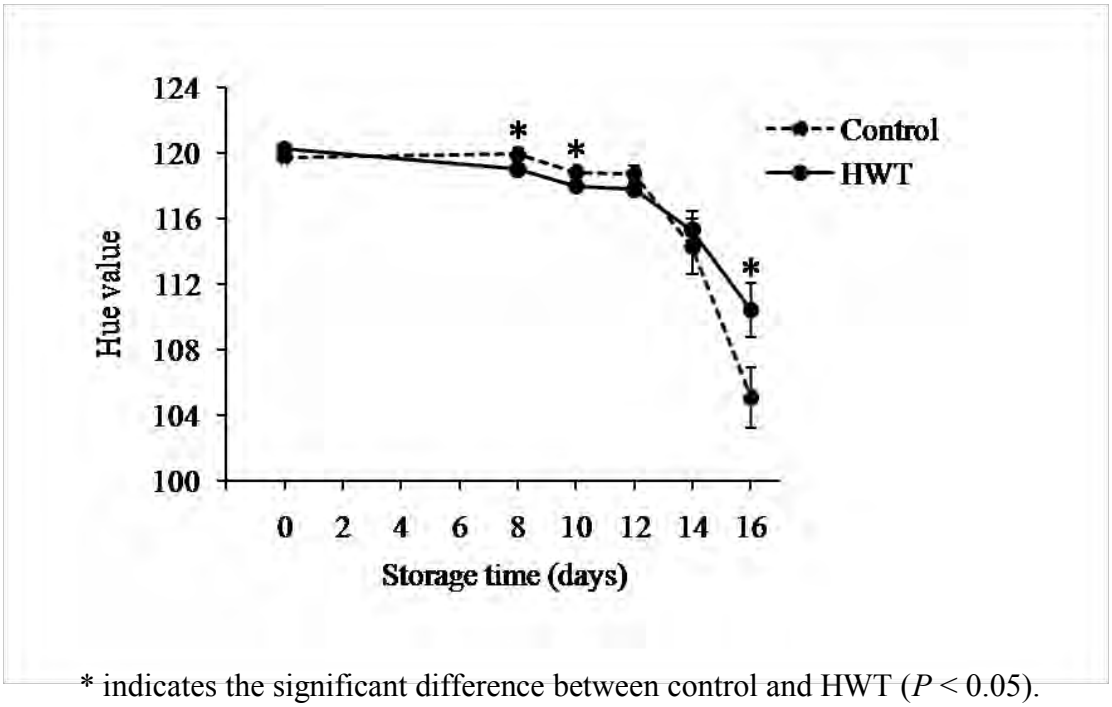


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.17 L value of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.2 Hue value

Hue value tended to decrease during storage in both treatments. A significantly higher hue value in control banana was observed on day 8 and 10. However, on day 16, hot water treated banana fruits showed significantly higher Hue value (Figure 4.18).

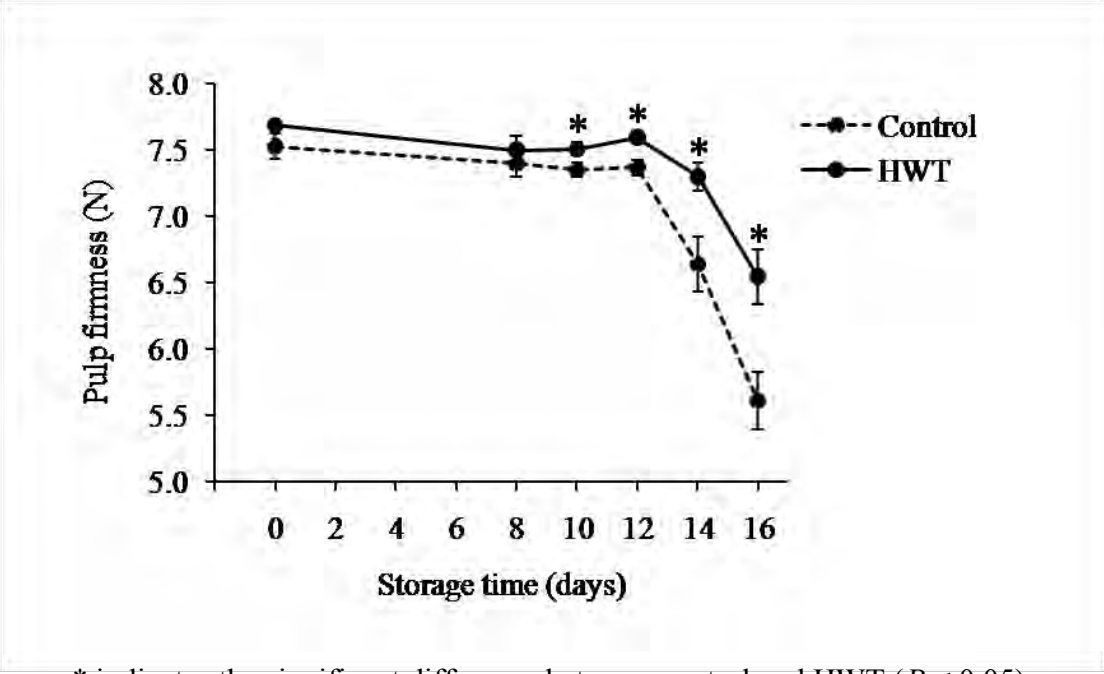


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.18 Hue value of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.3 Pulp firmness

During storage at 14°C for 8 days, the pulp firmness of hot water treated banana showed a higher value than those of control, and then maintained a relatively higher level upon ripening at 25°C. The significant differences were shown on day 10, 12, 14 and 16 (Figure 4.19). Hue values were correlated with pulp firmness in both control (r = 0.995, $P < 0.01$) and hot water treated fruits (r = 0.970, $P < 0.01$).



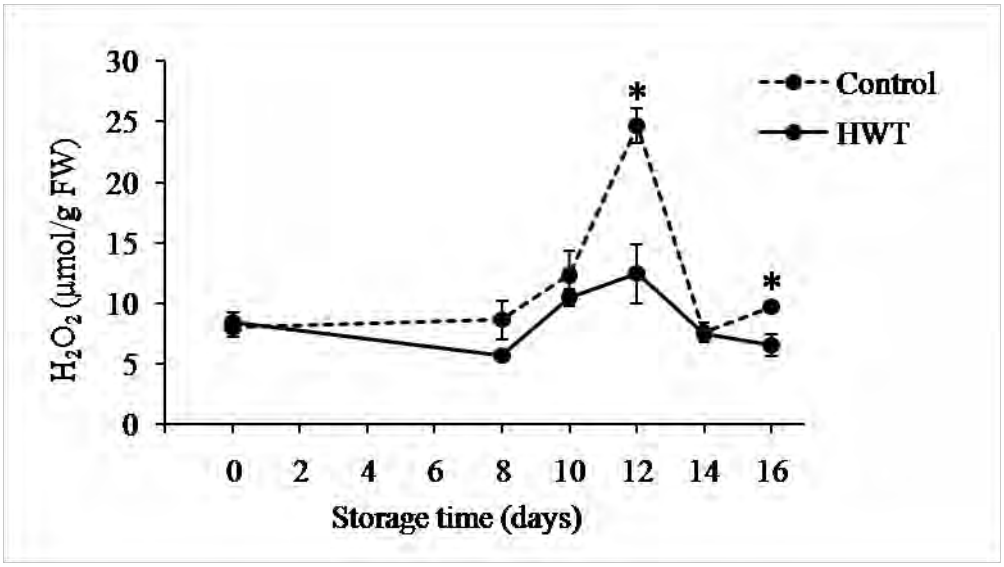
* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.19 Pulp firmness of banana during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.4 H_2O_2 determination

1.2.4.1 H_2O_2 concentration

Drastic increases of H_2O_2 content in control banana were found from day8 to day12, although, it decreased on day 14. Hot water treated banana tended to have a lower H_2O_2 content than those of control. The significant differences were found on day 12 and day 16 (Figure 4.20).



* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.20 H_2O_2 contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.4.2 H_2O_2 staining

The contents of H_2O_2 in hot water treated banana tended to be lower than that of control on day 8, day 10 and day 12 (Figure 4.21). After day 12 the signal of fluorescence was higher in hot water treatment as shown on day 14 and day16 (Figure 4.22). Overall, the H_2O_2 contents tended to decrease during storage as measured by lower fluorescence signals in both treatments. These results contradict with the H_2O_2 profiles measured by spectrophotometric assay, which showed an increase in H_2O_2 during storage. Higher H_2O_2 contents were also measured by the spectrophotometric assay in control fruit throughout storage time, including during ripening period on day 16.

(A)

(B)

(C)

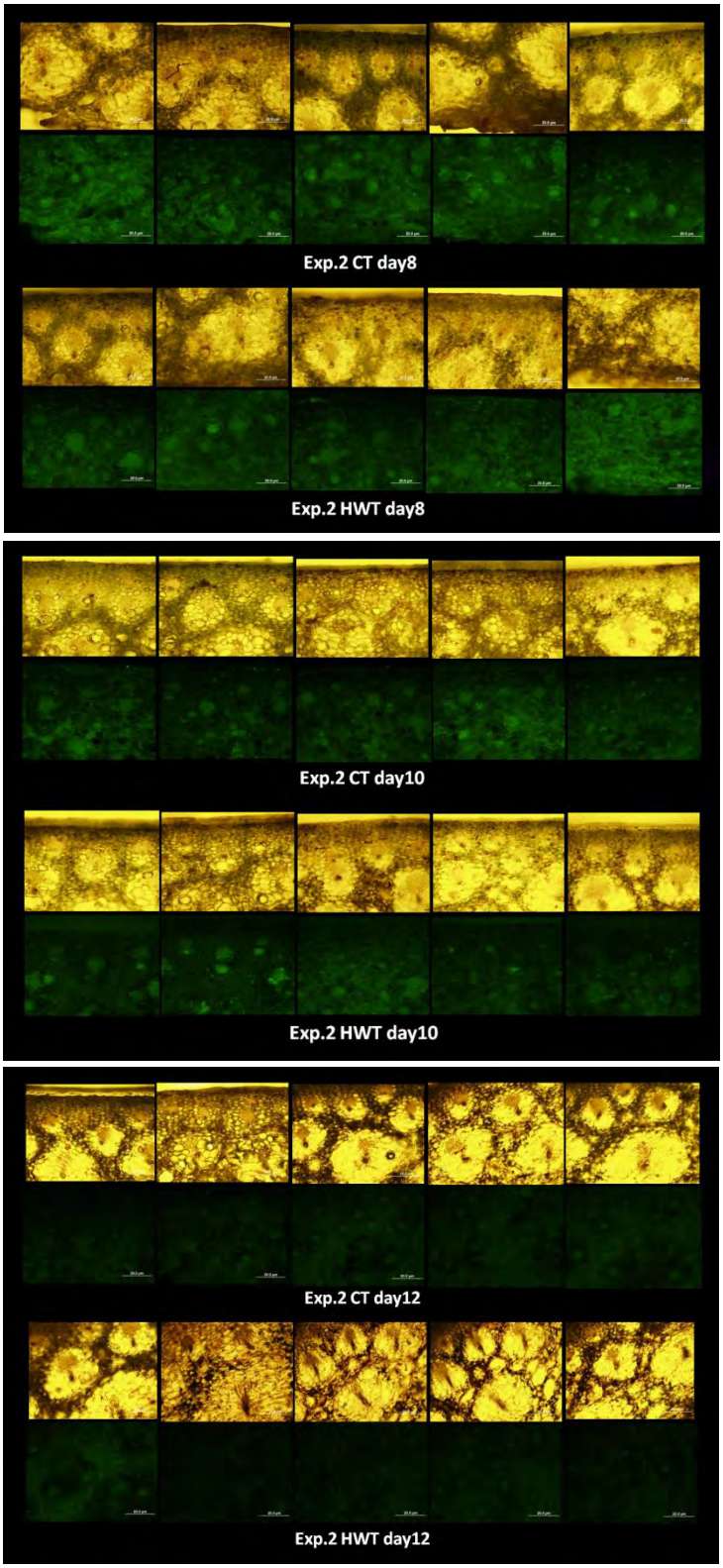
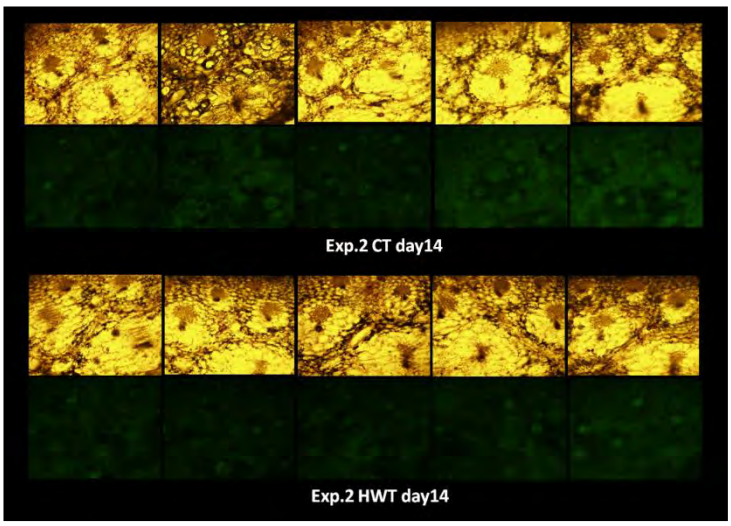


Figure 4.21 H_2O_2 staining of banana peel during storage at 14 °C for 8 days, followed by storage at 25 °C. The upper rows of each treatment show the normal peel tissue under light microscope: the lower rows show the stained tissue with H_2DCFDA. (A) – (C) show the H_2O_2 staining for day 8, 10 and 12, respectively.

(D)



(E)

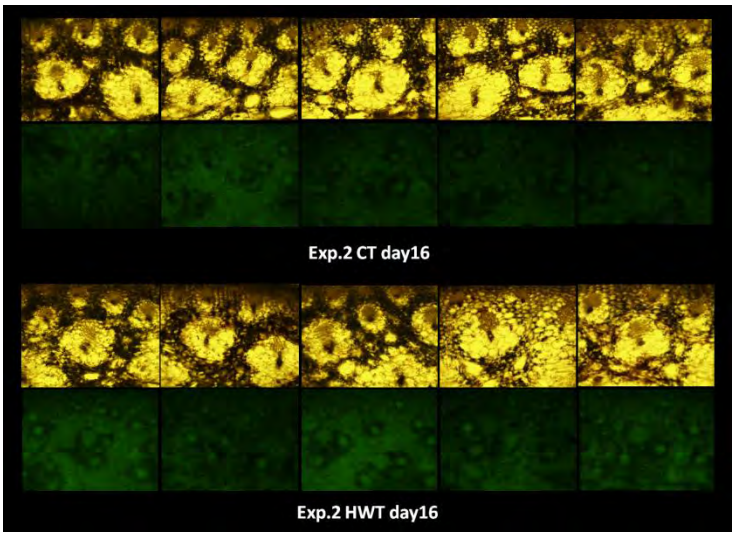
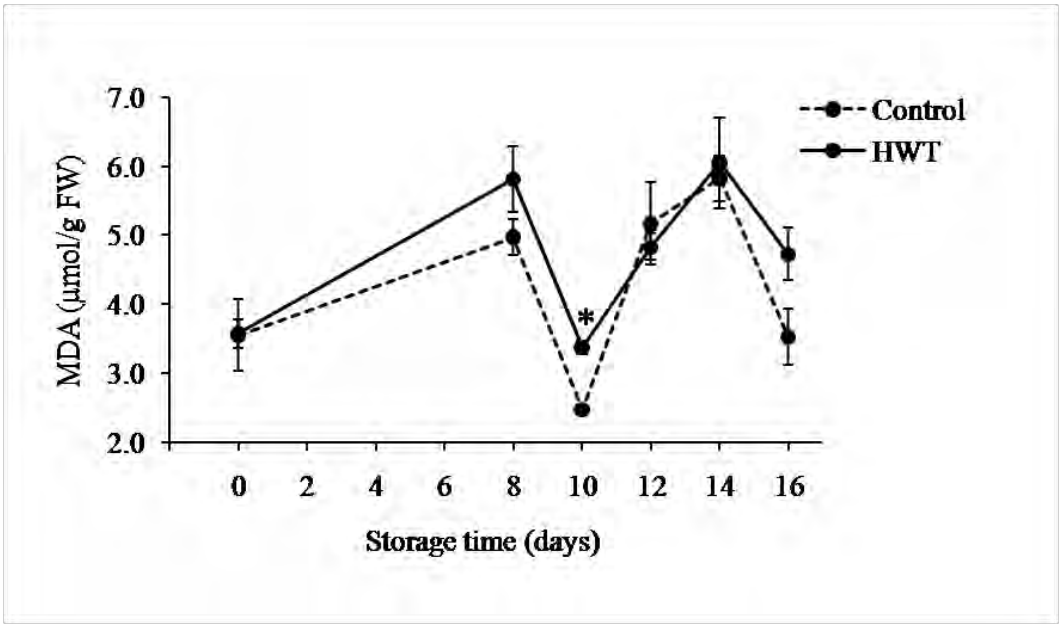


Figure 4.22 H_2O_2 staining of banana peel during storage at 14 °C for 8 days, followed by storage at 25 °C. The upper rows of each treatment show the normal peel tissue under light microscope, the lower rows show the stained tissue with H_2DCFDA. (A) – (E) show the H_2O_2 staining for day 8, 10, 12, 14 and 16, respectively.

1.2.5 MDA concentration

MDA content increased after stored at 14°C for 8 days and dropped dramatically on day 10. Subsequent measurement, indicate a sharp increase from day 12 to day 14, and then a decrease on day 16. Hot water treated banana fruit showed a tendency for higher MDA content than those of control. The significant difference was found on day 10 (Figure 4.23).

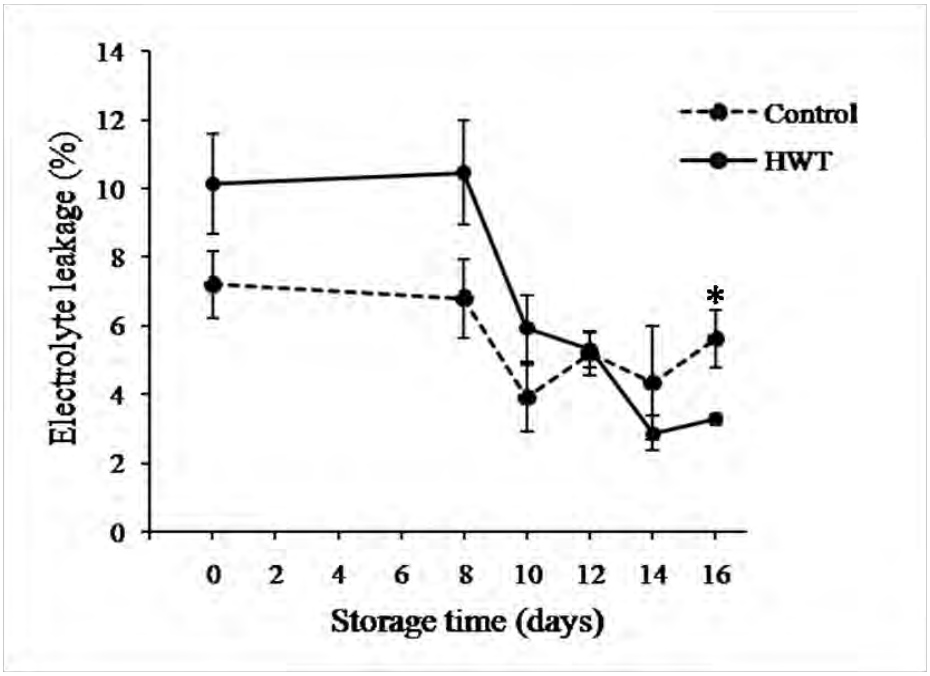


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.23 MDA contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.6 Electrolyte leakage

The percentage of electrolyte leakage in hot water treated fruit was higher than those in control fruit on day 0, 8 and 10. When fruits were transferred to ripen at 25 °C (on day 8), the electrolyte leakage decreased to a lower level than those observed on day 8 and slightly increased when fruits were ripening. The control fruits showed higher electrolyte leakage than those of the hot water treatment during day 14 to day 16, significant differences could be found on day 16 (Figure 4.24).

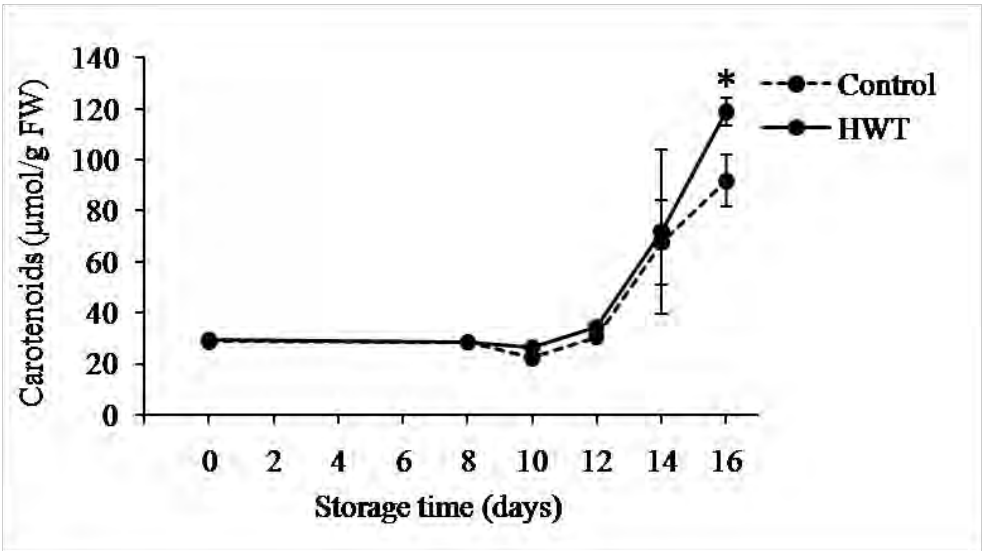


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.24 Electrolyte leakage of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.7 Carotenoid concentration

Following the removal of banana fruit from 14 °C storage, carotenoid contents increased continuously in both treatments. Hot water treated banana fruit showed a higher carotenoid content than those of control. The significant difference of carotenoid contents was found on day16 (Figure 4.25).

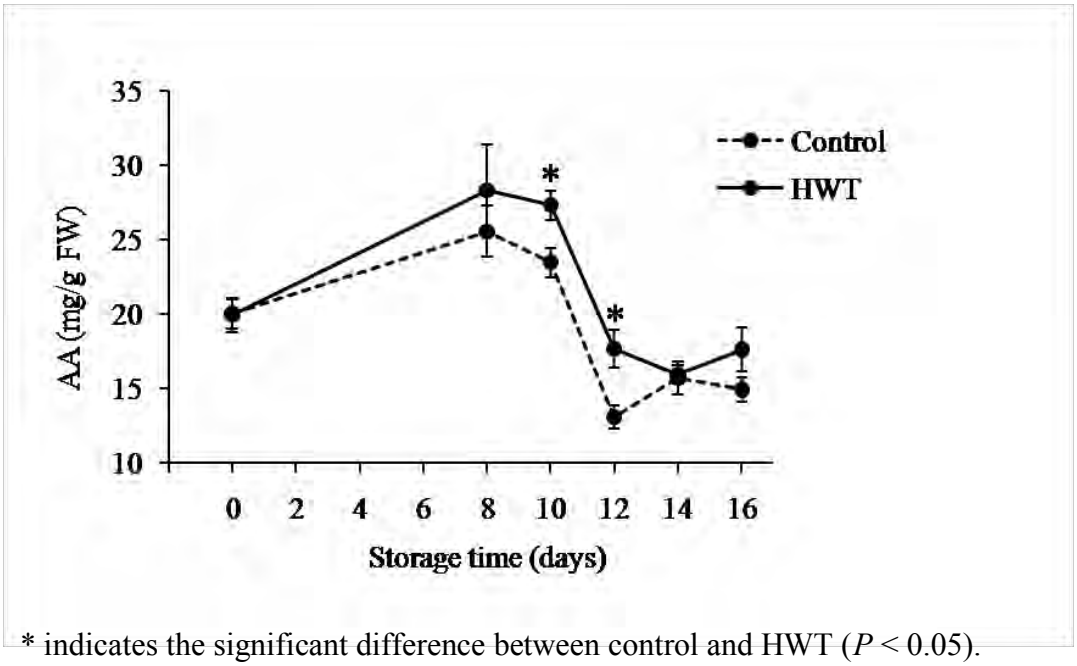


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.25 Carotenoid contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.8 Ascorbic acid concentration

The AA contents increased after being stored at 14 °C for 8 days but rapidly decreased upon transfer to 25°C. Hot water treated banana fruit tended to have higher AA contents than those of control. The significant differences were shown on day 10 and day 12 (Figure 4.26).

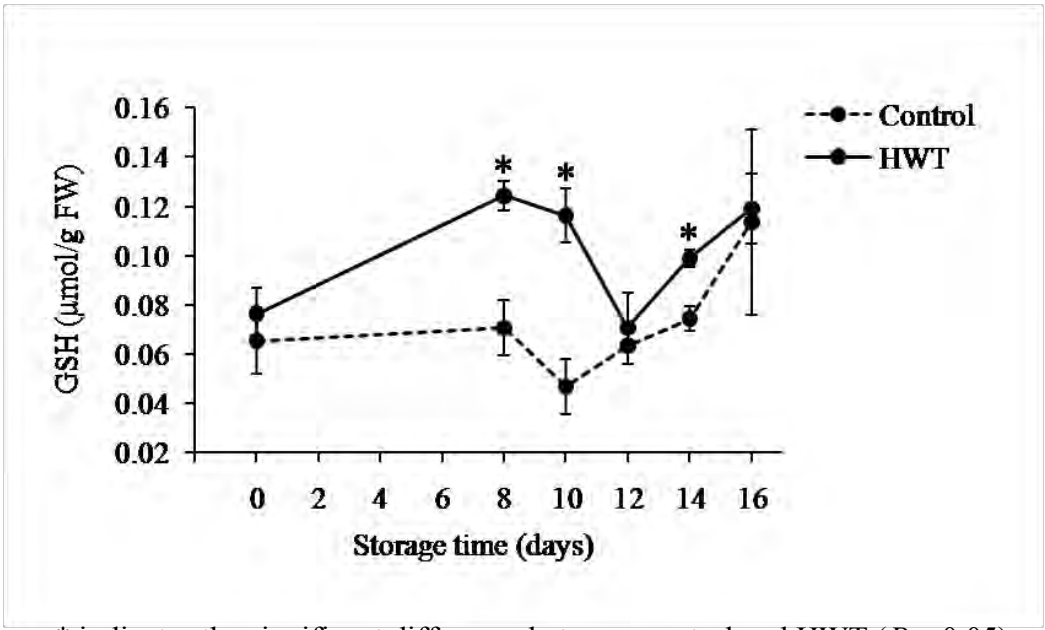


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.26 Ascorbate contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.9 GSH concentration

In the hot water treatment, GSH contents increased on day 8, and then dropped on day 10 and day 12. Subsequent measurements showed increases in GSH contents during ripening on day 14 and day 16. In the control treatment, GSH contents were stable from day 0 to day 8, then, slightly decreased on day 10. GSH contents in both control and hot water treated treatment rapidly increased from day 12 to day 16. Hot water treated banana tended to have higher GSH than those of control. The significant differences could be found on day 8, 10 and 14 (Figure 4.27). In control fruit, GSH contents were correlated with carotenoid contents ($r = 0.899$, $P < 0.05$) but negatively correlated with hue value ($r = -0.904$, $P < 0.05$) and pulp firmness ($r = -0.901$, $P < 0.05$).

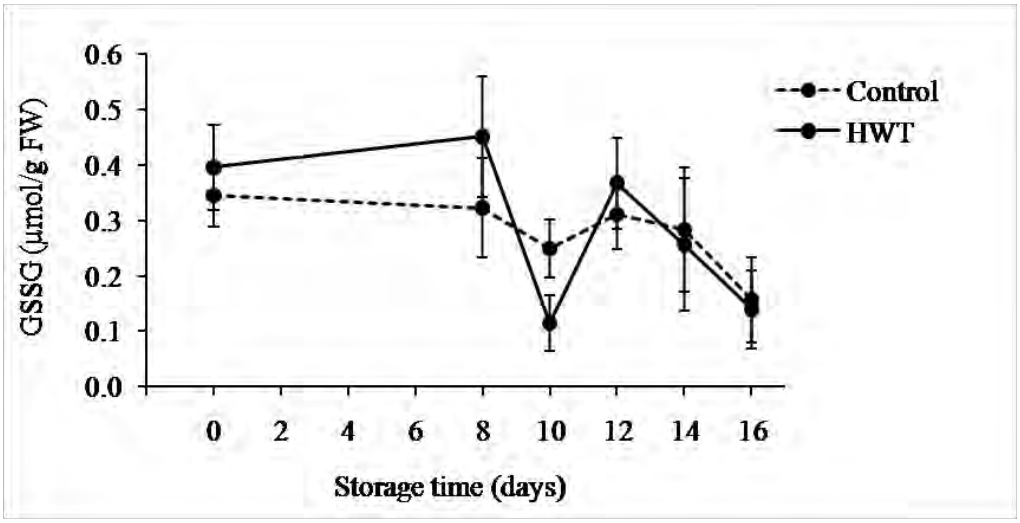


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.27 GSH contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.10 GSSG concentration

In control fruit, GSSG content tended to be stable from day 0 to day 8, then decreased on day 10 and day 16. In hot water treatment, GSSG contents increased on day 8 and then decreased on day 10. After that, they were increased on day 12, followed by decreasing until day 16. Hot water treated banana showed the tendency of the higher GSSG content on day 0, 8 and 12. However, there was no significant difference (Figure 4.28). In control fruit, GSSG contents were correlated with hue value (r = 0.889, $P < 0.05$) and pulp firmness (r = 0.878, $P < 0.05$).

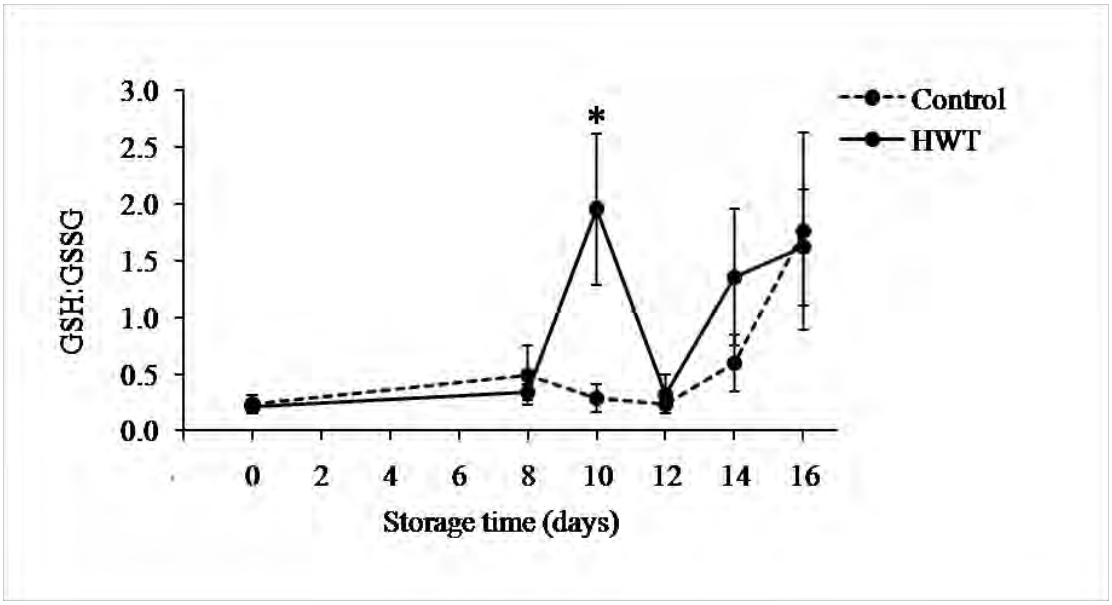


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.28 GSSG contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.11 GSH/GSSG ratio

During storage at low temperature (day 0 to day 8), the GSH/GSSG ratio was relatively stable in both treatments. On day 10 (after transfer to 25°C for 2 days), the GSH/GSSG measured in the fruits from the hot water treatment increased dramatically. The GSH/GSSG ratio decreased on day 12, then, sharply increased on day 14 and day 16. In control fruit, GSH/GSSG exhibited a small change from day 0 to day 12, then, rapidly increased from day 14 to day 16. The significant difference was found on day 10, which in hot water treatment showed the higher ratio of GSH/GSSG (Figure 4.29). In control fruit, GSH/GSSG ratio was correlated with carotenoid ($r = 0.899$, $P < 0.05$) and GSH ($r = 0.943$, $P < 0.01$) contents, while negatively correlated with hue value ($r = -0.963$, $P < 0.01$), pulp firmness ($r = -0.960$, $P < 0.01$) and GSSG contents ($r = -0.866$, $P < 0.05$). In hot water treated fruit, GSH/GSSG ratio was negatively correlated with GSSG contents ($r = -0.963$, $P < 0.01$).

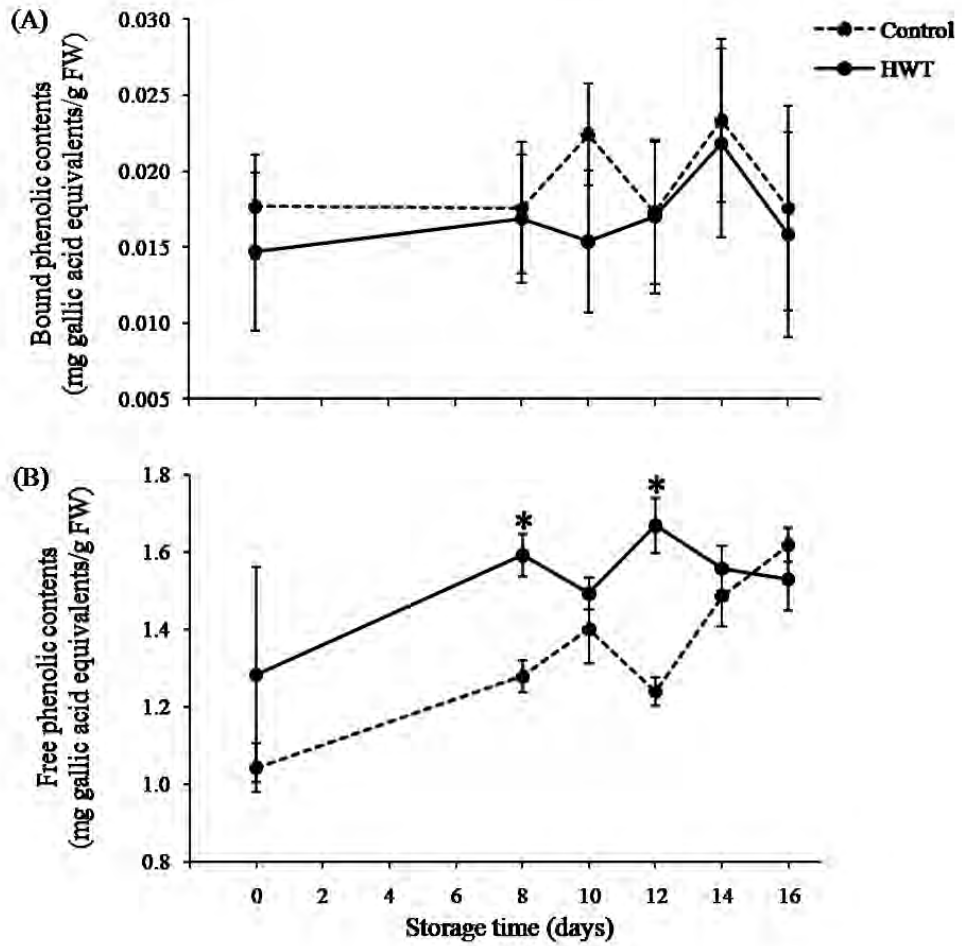


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.29 The ratio of GSH/GSSG of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.12 Bound and free phenolic compound concentrations

There were no significant differences of bound phenolic contents between control and hot water treatments. However, hot water treated fruits tended to have lower bound phenolics than those of control (Figure 4.30A). Free phenolics increased slightly during fruit ripening. Hot water treated fruits showed a higher value than those of control throughout storage, except on day 16. The significant differences were shown on day 8 and day 12 (Figure 4.30B). In control fruit, free phenolic contents were negatively correlated with hue value (r = -0.813, $P < 0.05$), pulp firmness (r = -0.847, $P < 0.05$) and GSSG contents (r = -0.884, $P < 005$).

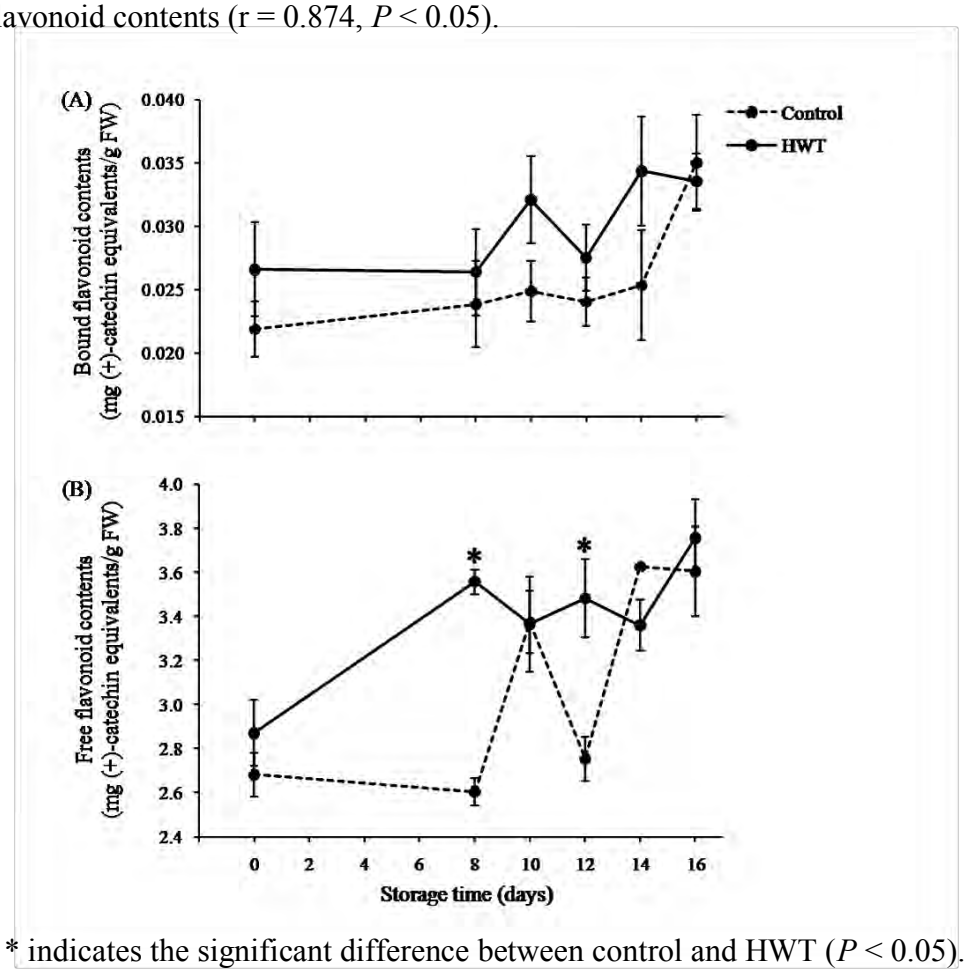


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.30 Bound (A) and free (B) phenolic contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.13 Bound and free flavonoid concentration

The bound flavonoids of banana tended to be higher in ripening fruit than in unripe fruit. Throughout storage time, hot water treated bananas demonstrated higher levels of bound flavonoids than those of control, except on day 16. However, significant differences could not be found (Figure 4.31A). In control fruit, bound flavonoid contents were correlated with carotenoid (r = 0.843, $P < 0.05$), GSH (r = 0.859, $P < 0.05$), GSH/GSSG (r = 0.971, $P < 0.01$) and free phenolic contents (r = 0.816, $P < 0.05$), but negatively correlated with hue value (r = -0.955, $P < 0.01$), pulp firmness (r = -0.942, $P < 0.01$) and GSSG contents (r = -0.947, $P < 0.05$). In hot water treated fruit, bound flavonoid contents were correlated with GSH/GSSG ratio (r = 0.885, $P < 0.05$), but negatively correlated with GSSG (r = -0.876, $P < 0.05$) and electrolyte leakage (r = -0.891, $P < 0.05$). Hot water treated fruit display higher free flavonoid contents than those of control, except on day14. Ripe fruit tended to have higher free flavonoids than unripe fruit in both control and hot water treatments. The significant differences of free flavonoids between the treatments were found on day 8 and day 12 (Figure 4.31B). In control fruit, free flavonoid contents were correlated with free flavonoid contents (r = 0.874, $P < 0.05$).



* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.31 Bound (A) and free (B) flavonoid contents of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.2.14 DPPH radical radical scavenging activities in bound and free extracts

The DPPH radical scavenging activities of bound extracts of bananas tended to decrease during storage. The highest levels of antioxidant activities were found on day 0, and then decreased during ripening. Except on day12, Hot water treated banana tended to have higher levels of antioxidant activities than those of control. However, there were no significant differences between the treatments (Figure 4.32A). In control fruit, DPPH radical scavenging acitivity in bound extracts were correlated with H_2O_2 contents ($r = 0.909$, $P < 0.05$). However, in hot water treated banana, the DPPH radical scavenging acitivity in bound extracts were negatively correlated with free phenolic contents ($r = -0.906$, $P < 0.05$). The antioxidant activities of free extracts in both control and hot water treatment decreased during ripening. Hot water treated bananas tended to have higher antioxidant activity than those of control from day 8 to day 16. However, significant differences could not be found (Figure 4.32B). In control fruit, DPPH radical scavenging activity in free extracts were negatively correlated with free phenolic contents ($r = -0.826$, $P < 0.05$). In hot water treated fruit, DPPH radical scavenging activity in free extracts were correlated with those in bound extracts ($r = 0.963$, $P < 0.01$), but negatively correlated with free phenolic ($r = -0.852$, $P < 0.05$) and free flavonoid ($r = -0.861$, $P < 0.05$) contents.

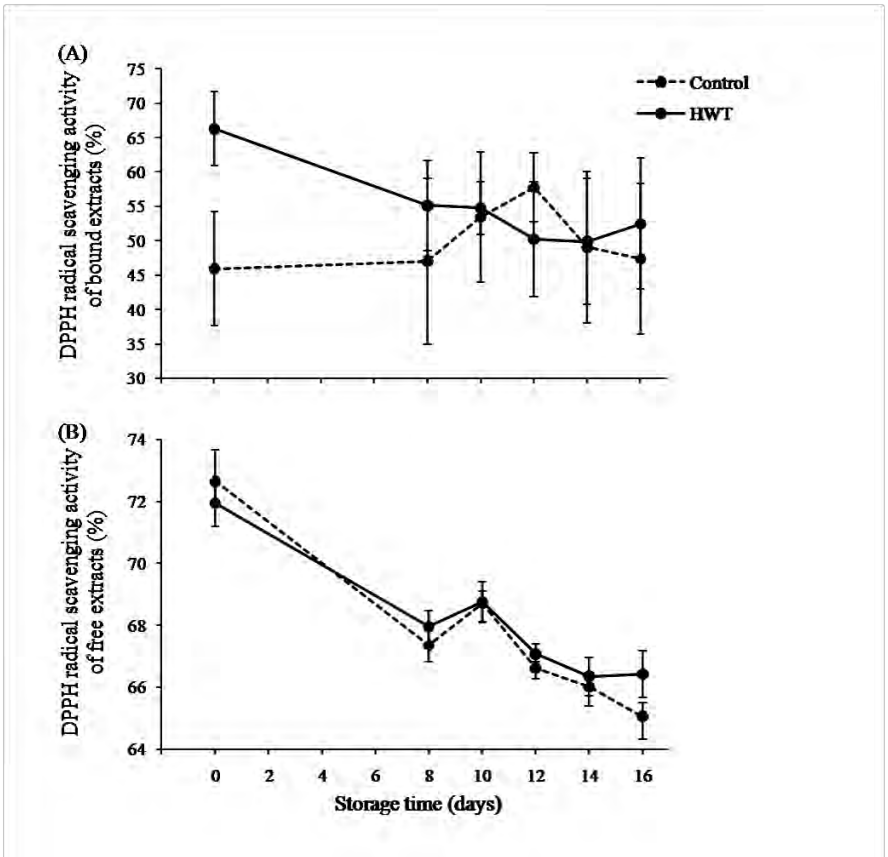


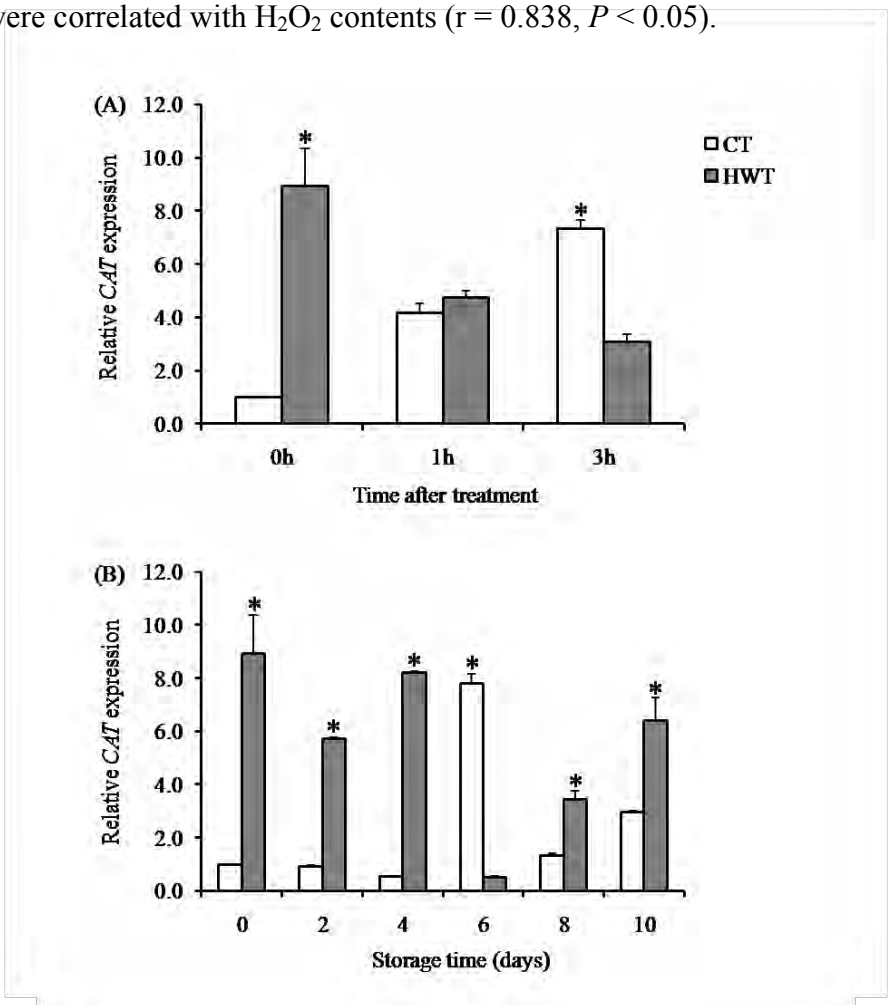
Figure 4.32 DPPH radical scavenging activity in bound (A) and free (B) extracts of banana peel during storage at 14°C for 8 days, followed by storage at 25°C.

1.3 Effects of hot water treatments on antioxidant gene expressions.

1.3.1 Effects of hot water treatments on *CAT* and *SOD* expression during storage at 25 °C.

1.3.1.1 The expression of *CAT* during storage at 25 °C.

The expression of *CAT* increased immediately after hot water treatment (0 h), then decreased to the level of control (CT) after 1 h, and then finally to a lower level at 3 h after treatment (Figure 4.33A). However, on day 2 and day 4, *CAT* expression in hot water treated fruit were higher than those of control. Also, during fruit ripening on day 8 and day 10, hot water treatment showed higher *CAT* expression, during a period when the ripening of hot water treated banana was delayed (Figure 4.33B). During storage of the hot water treated fruit, CAT expressions were correlated with H_2O_2 contents ($r = 0.838$, $P < 0.05$).

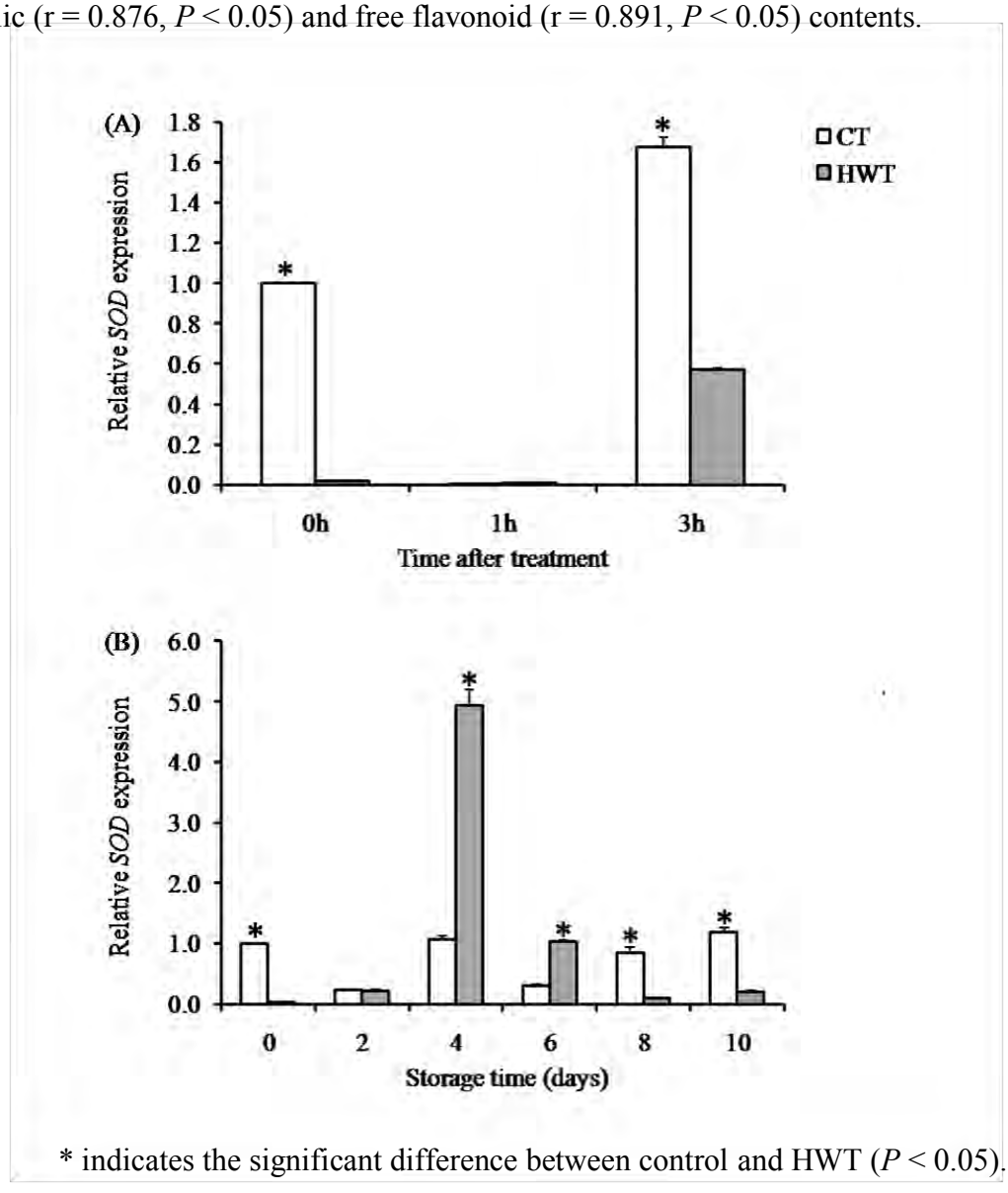


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.33 The relative ratio of *CAT* expression in banana peel during storage at 25 °C. (A) The expression of *CAT* after treatment. (B) The expression of *CAT* after storage at 25 °C.

1.3.1.2 The expression of *SOD* during storage at 25 °C.

The activation of *SOD* expression by hot water treatment was observed on day 4 and day 6 after storage. While on subsequent days, including the period of ripening on day 8 and day 10, banana fruits in control showed higher *SOD* expression (Figure 4.34B). In control fruit, SOD expressions were negatively correlated with MDA contents (r = -0.485, $P < 0.05$) during storage. In hot water treated fruit, SOD expressions were correlated with electrolyte leakage (r = 0.881, $P < 0.05$), free phenolic (r = 0.876, $P < 0.05$) and free flavonoid (r = 0.891, $P < 0.05$) contents.



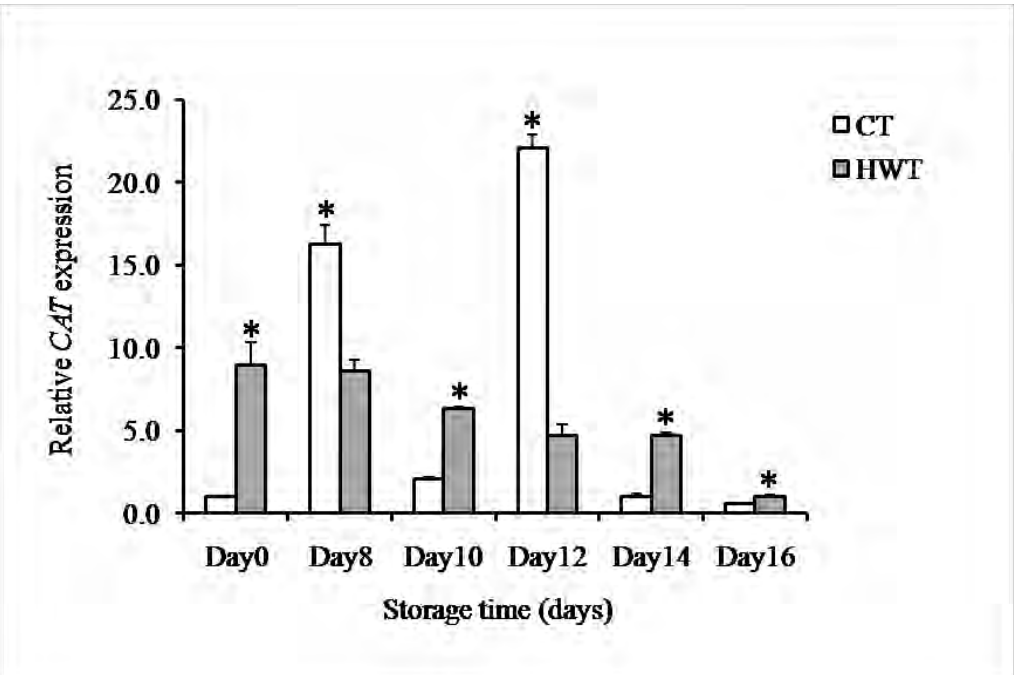
* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.34 The relative ratio of *SOD* expression in banana peel during storage at 25 °C. (A) The expression of *SOD* after treatment. (B) The expression of *SOD* after storage at 25 °C.

1.3.2 Effects of hot water treatments on *CAT* and *SOD* expression during storage at 14 °C for 8 days, followed by storage at 25 °C.

1.3.2.1 The expression of *CAT* during storage at 14 °C for 8 days, followed by storage at 25 °C.

After storage at 14 °C for 8 days, the expression of *CAT* in control fruits showed an increase to a higher level than that of hot water treated fruits, then a decrease to a lower level on day 10. Although on day 12, higher *CAT* expressions were found in control, and during ripening on day 14 and day 16, higher *CAT* was observed in the hot water treatment (Figure 4.35). In hot water treated fruit, *CAT* expressions were correlated with hue value ($r = 0.941$, $P < 0.01$), pulp firmness ($r = 0.848$, $P < 0.05$), electrolyte leakage ($r = 0.885$, $P < 0.05$), but negatively correlated with carotenoid contents ($r = -0.867$, $P < 0.05$).

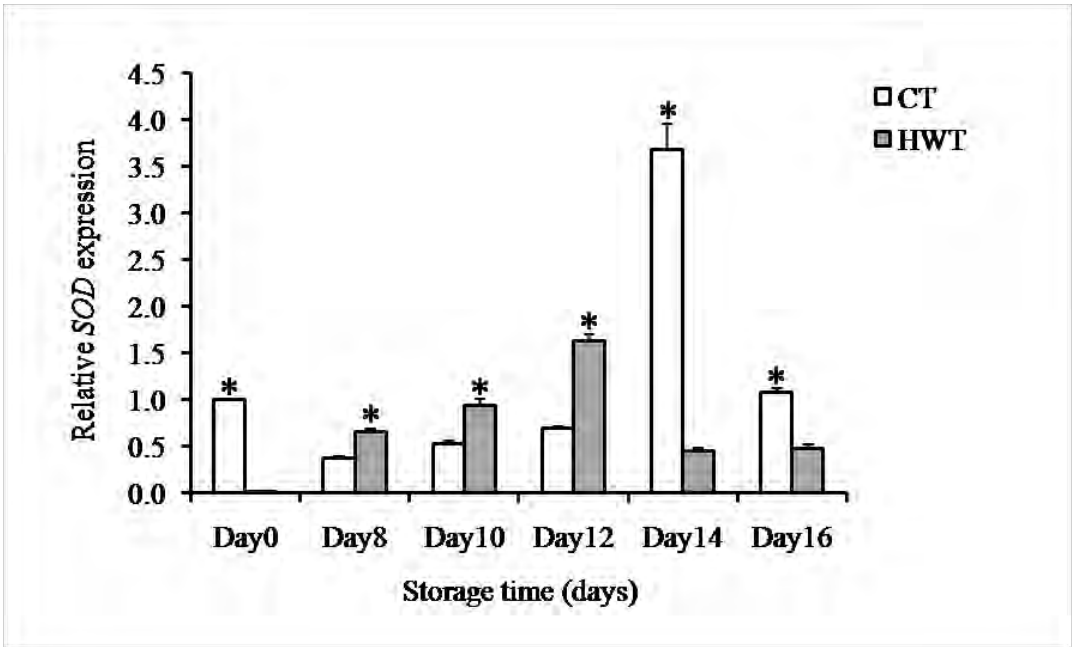


* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.35 The relative ratio of *CAT* expression in banana peel during storage at 14 °C, followed by storage at 25 °C.

1.3.2.1 The expression of *SOD* during storage at 14 °C for 8 days, followed by storage at 25 °C.

Higher *SOD* expression was measured in hot water treated fruits after removing fruits from 14 °C on day 8 and during storage at 25 °C on day 10 and day 12. However, during fruit ripening on day 14 and day 16, higher *SOD* expressions were found in control fruits (Figure 4.36).



* indicates the significant difference between control and HWT ($P < 0.05$).

Figure 4.36 The relative ratio of *CAT* expression in banana peel during storage at 14 °C, followed by storage at 25 °C.

2. DISCUSSION

2.1 Effects of hot water treatment on fruit quality (L value, hue value, and pulp firmness)

For banana fruits stored at 25 °C for 10 days (*Experiment 1*), the changes in external quality were observed by measuring peel color (L and hue value) and pulp firmness. The ripening of fruit was shown to occur during day6 to day10 after storage, which related to the increase in L value, the decrease of hue value and the decrease of pulp firmness. These changes suggest that hot water treatment can delay of ripening during storage at 25 °C.

For bananas stored at 14 °C for 8 days before being transferred to 25 °C (*Experment 2*), the development of ripening was not different among the treatments until full ripening was reached in control fruit on day16. Hot water treatment tended to delay ripening by slowing the increase in L value, and decreasing of hue value and pulp firmness, as observed in *Experiment1*.

The storage of fruit is accompanied by a loss of cell-wall integrity due to the breakdown of pectin substances leading to an increase in soluble pectin and a decrease in fruit firmness. In pomegranate, hot water treatment induced increases in free putrescine and spermidine during storage, which could have a role in the lower rate of fruit softening (Mirdehghan et al., 2007). In strawberry, heat treatment can delay fruit softening by reducing endo-1, 4-β-D-glucanase, β-galactosidase activities, while pectimethyl esterase activity was enhanced. This broad effect of heat treatments on cell-wall degrading enzymes could slow down pectin solubilisation by reducing pectin cleavage and by increasing the amount of putative sites for calcium bridge formation in the cell walls (Vicente et al., 2006). Moreover, the higher polyamine concentration could account for the greater firmness retention in heat-treated fruit (Vicente et al., 2006).

2.2 Effects of hot water treatment on the concentration of H_2O_2 and MDA, and electrolyte leakage

In *Experiment 1*, according to the level of ROSs in cells, hot water treated banana showed a lower concentration of H_2O_2 than that of control during ripening. This result was related to the level of lipid peroxidation indicated by MDA concentration, which were lower in hot water treated fruits. The percentage of electrolyte leakage in both treatments was not different during day0 to day6, although, hot water treated banana tended to have higher electrolyte leakage than that of control fruit. However, lower electrolyte leakage in hot water treatment was found during fruit ripening, with significant difference shown on day8. This result suggests that hot

water treatment could increase electrolyte leakage just in the early period after treatment, whereas during later period hot water treatment could reduce the electrolyte leakage to a lower level than that of control. The lowering of H_2O_2 found in hot water treatment may have some beneficial effects on the reduction of membrane damage indicated by electrolyte leakage and MDA contents.

In *Experiment 2*, according to H_2O_2 content, MDA, and electrolyte leakage, hot water treatment tended to reduce the H_2O_2 concentration during storage. However, the MDA contents in hot water treated fruits were higher than those in control fruits. The increase of MDA in hot water treated fruits was related to an increase in electrolyte leakage, although the reduction of leakage was detected in hot water treatment during ripening on day 14 to day16. This result may suggest that hot water treatment could induce an increase in membrane leakage during early periods of storage, but decrease when fruit ripens.

Surassawadee Promyou et al. (2008) found the effects of hot water treatment on the delaying of peel blackening during cold storage (4°C) in 'Hom Thong' (cv. Gros Michel) banana. This effect was related with an increase in the ratio of unsaturated to saturated fatty acids, a decrease of LOX activity, and also a reduction of membrane degradation (MDA).

The increase in membrane permeability can have an effect on the increase in ion leakage, which is associated with chilling of sensitive tissue (Saltveit, 2000). The development of chilling injury in pomegranate was highly correlated to the skin browning and EC leakage (Mirdehghan et al., 2007).

Hot water treatment, which resulted in no visible injury to the fruit, can enhance chilling tolerance by slowing the reduction of electrolyte leakage. Recent studies suggested that hot water treatment can induce the production of heat shock proteins (Klien and Lurie, 1991; Lafuente et al., 1991; Surassawadee Promyou et al., 2008) and that these proteins confer enhanced tolerance to chilling temperatures.

There were some reported found that hot water immersion could reduce electrolyte leakage. In cucumber fruit, hot air treatment can reduce CI, respiratory rates and electrolyte leakage, compared with non-heated fruit (Hirose, 1985). However, McCollum et al. (1995) found that electrolyte leakage increased immediately in cucumber fruit after heat treatment with no significant difference among the treatments.

Heat-treated apple showed an increase in membrane microviscosity and sterol content while phospholipid fatty acid became more saturated. The electrolyte leakage also increased from apple fruit disc. However, the microviscosity and leakage were lower in heated fruit, and increased more slowly during ripening at 20 °C than in control apples (Lurie et al., 1995).

In pomegranate, hot water treatment showed lower electrolyte leakage, which correlated with an increase of polyamine and a higher maintenance of the unsaturated/saturated fatty acid ratio (Mirdehghan et al., 2007). This result may suggest the effect of hot water treatment on inducing tolerance mechanism of pomegranate fruit to low temperature by the maintenance of membrane integrity and fluidity.

2.3 Efects of hot water treatment on carotenoid and ascorbic acid concentrations

In *Experiment 1*, regarding antioxidant compounds, including carotenoids, ascorbates, glutathione, phenolics and flavonoids, it was found that hot water treatment had some effects on these compounds during storage. Carotenoids tended to be higher in hot water treatment during fruit ripening, while ascorbate contents tended to higher in hot water treatment at an earlier period of storage.

In *Experiment 2*, according to the analysis of the carotenoids and ascorbates contents, hot water treatment showed minimal effect on these contents during the progress of ripening. However, higher level of both carotenoids and ascorbate contents could be found in later period of ripening.

Carotenoids are one of the compounds contributing to antioxidant activity in plant (Babbar et al., 2011). The increases of carotenoids after hot water treatment were also found in mango fruit (Talcott et al., 2005; Djioua et al., 2009).

Ascorbates could possibly contribute to the total antioxidant activity (Babbar et al., 2011). In most fruits and vegetables, the ascorbic acid content was relatively stable during storage Kervers et al. (2007). However, a decrease of ascorbic during storage had been reported in guava (Bashir et al., 2003), apricot, banana, spinach, melon, cherry, citrus and leek Kervers et al. (2007). Strawberries, bananas, lemons and oranges, which showed high antioxidant capacity, have also high level of ascorbic acid (Kervers et al., 2007). According to hot water treatment, it was found that hot water treated mango showed an increase of ascorbate (vitamin C) content during storage (Djioua et al., 2009)

2.4 Effects of hot water treatment on the concentration of GSH and GSSG and the GSH/GSSG ratio

In *Experiment 1*, reduced glutathione (GSH) showed higher levels in hot water treated fruit throughout the storage time; while oxidized glutathione (GSSG) were lower in hot water treatment until ripening on day10. Based on the glutathione contents, hot water treatment tended to have higher GSH/GSSG ratio than that of

control, indicating a more active form of glutathione content in hot water treated fruits.

For the glutathione profiles in *Experiment 2*, the GSH contents in hot water treatment increased 1.7- and 2.5-folds higher than those in control on day8 and day10, respectively. Increases in GSH were observed in ripe banana of both treatments, while the GSSG contents decreased. Higher GSH/GSSG ratio in hot water treatment was shown during development at 25 °C. However, a significant difference was found only in unripe fruits on day10. From the results of the glutathione level, it is suggested that hot water treatment could increase the level of GSH at an early storage period after removal to 25 °C, and then the patterns of glutathione changes were unaffected. As a result, the GSH/GSSG ratio was higher in hot water treatment as well as the GSH level in hot water treated fruit before ripening started.

GSH has an important function in maintaining cellular redox status and plays a significant role in antioxidant protection (Noctor and Foyer, 1998). The reduction in the endogenous level of GSH might be its utilization as a reducing substrate in the synthesis of ascorbic acid (Cai et al., 2011). Increase of GSH content might contribute to reduce oxidative levels in cells.

2.5 Effects of hot water treatment on the concentrations of phenolic compounds, flavonoids, and antioxidant capacity

According to phenolic and flavonoid concentrations, higher contents were found in free extracts (soluble or hydrophilic phase) than in bound extracts (insoluble or lipophilic phase). In *Experiment 1*, bound phenolics tended to be stable in both control and hot water treatment throughout fruit development with no significant difference. While, for free phenolics, hot water treated fruit showed a rapid increase (2-folds to that of control fruit) on day4 and then decreased to a steady level during the progression of ripening. Bound flavonoids showed a small increase during storage with no significant difference, but in free extracts, hot water treated fruit had a higher level of flavonoids during the beginning stages of ripening. The increase of free phenolics and flavonoids at the beginning of fruit development through hot water treatment may have an effect on later ripening processes of banana during storage. However, the changes of free phenolics and flavonoids were not correlated with the changes of antioxidant capacity measured by DPPH method, which showed a steady level in bound extracts and a continuous decrease in free extracts. This lack of correlative result may be due to some active antioxidant compounds in this banana cultivar that could not be measured by the reaction. As a result, the total antioxidant capacity was not accounted for from some phenolics or flavonoids in the samples.

In *Experiment 2*, according to the phenolics, flavonoids and antioxidant capacity, it was found that both phenolics and flavonoids increased during storage. Significant differences were observed in free extracts, where higher contents were shown in hot water treatment. However, in contrast, the antioxidant capacity gradually decreased in both control and hot water treatment during storage. Hot water treatment tended not to have an affect on the antioxidant capacity in both bound and free extracts of banana.

The decrease of phenolic contents during fruit ripening were also found in medlar (Ayaz et al., 2008), blueberry (Castrejon et al., 2008), guava (Bashir et al., 2003), acerola fruits (*Malpighia emarginata* D.C.) (Lima et al., 2005), pear fruit (Amiot et al., 1995) and apple fruits (Burda et al., 1990).

There are reports about a transient increase of phenolic compounds during storage in plum, tomato, broccoli, and black grape, while a transient decrease was observed in citrus, lettuce and celery. In banana, the phenolic contents decrease rapidly (only 20% was still present after 2 days) (Kervers et al., 2007). In blueberry, different cultivation and climate condition effect total phenolics (Castrejon et al., 2008). Gruz et al. (2011) reported that the amount of phenolic acids decreased during the ripening progressed, while the insoluble ester-bound phenolics showed an increase during early stages of maturity and then decreased during the ripening development until over-ripe. In tomato, levels of aqueous phenolics increased during ripening (Cano et al., 2003). In mango, a significant decrease of total soluble phenolics was found during development from mature green to full ripe stages (Kim et al., 2007). The phenolic contents in mango were unaffected by hot water treatment (Talcott et al., 2005; Kim et al., 2007). However, Kim et al. (2009) found that hot water treated mango showed a decline in total soluble phenolic. In banana, hot water treatments have no significant effects in the level of total free phenolics (Promyou et al., 2008). In kumquat (*Fortunella japonica* Lour. Swingle Cv. Ovale) fruit, the levels of total phenols decreased after hot water dip (Schirra et al., 2008).

Generally, the flavonoid content of fruits and vegetables increased during storage, or otherwise remained stable. In plum, for example, a transient increase in flavonol glycon and anthocyanin levels was reported (Kervers et al., 2007). In blueberry, anthocyanins increased during harvest stage, then a decrease of flavonols and hydroxycinnamic acids was observed during the development from unripe green to ripe blue stage (Castrejon et al., 2008).

The decrease of flavonoid contents were found in some studies. For instance, the flavonoids such as quercetin glycosides, phloridzin and chlorogenic acid, which was found in more abundant in the skin of apple than in the pulp, decreased during fruit development to reach a steady level during maturation and ripening (Awad et al., 2001). Also, a loss of the total flavonoid was found in banana and lettuce (Dupont et al., 2000). In banana, the flavonoid and aglycones decreased rapidly during storage

(Kervers et al., 2007). Castrejon et al. (2008) suggested that flavoniods biosynthesis is tightly associated with the development stage of fruit. According to hot water treatment, the levels of total flavonoids were increased in kumquat (*Fortunella japonica* Lour. Swingle Cv. Ovale) fruit after hot water dip (Schirra et al., 2008).

For most fruits and vegetables, storage did not negatively affect the antioxidant capacity except broccoli which showed a decrease of antioxidant capacity during storage (Kervers et al., 2007) and also in banana (Kondo et al., 2005) and apricot (Bartolini et al., 2006). The decrease in antioxidant activity was also found in medlar (*Mespilus germanica* L.) (Gruz et al., 2011). Antioxidant capacity decreased about 25% in apricot and decreased by more than 50% in spinach, banana, broccoli, and leek. In mango, antioxidant capacity significantly decreased throughout fruit ripening from mature-green to full ripe stages but were unaffected by the hot water treatment (Kim et al., 2007).

The transient increases in antioxidant capacity could be found in yellow pepper, asparagus and plum (Kervers et al., 2007). In tomatoes, levels of aqueous phenolics increased during ripening, which correlated with an increase of total antioxidant activity, however, this increase was mainly due to changes to the lipophillic antioxidant activity (hydrophilic antioxidant remained practically unchanged during ripening) (Cano et al., 2003). In orange and apple, the antioxidant doubled rapidly but afterward was stable. In onion, the antioxidant capacity continuously increased during storage (> 10 times after 23 days) (Kervers et al., 2007).

The correlation of total phenolic concentration and the DPPH radical scavenging activity was found in banana (Kondo et al., 2005). The significant correlation between total phenolic content and antioxidant activity measured with DPPH radical suggest that the presence of phenolic compounds largely accounted for the antioxidant capacity measured with DPPH (Kervers et al., 2007). However, correlations between phenolic compound and ORAC measurements were found in potatoes (Andre et al., 2007). Thus, the radical source used in the assay can have dramatic effects on the result because of differential responses of different types of antioxidant compounds to the radical source (Cao et al., 1996). There were some divergences existing between the results obtained from the ORAC assay and the DPPH method. For example, banana, green grapes and lemons, which have the highest total phenolic contents, had high antioxidant capacity as assayed by DPPH method, while plums, oranges and cherries had higher antioxidant capacity when assayed by the ORAC assay (Kervers et al., 2007).

2.6 Effects of hot water treatment on *CAT* and *SOD* expression

Several studies have been directed towards isolation and characterization of ripening-specific genes from banana. Consequently, genes encoding enzymes such as, pectate lyase (Domı´nguez-Puigjaner et al., 1997), polygalacturonase (Asif and Nath, 2005), ACC oxidase and catalase (Mbe´guie´-A-Mbe´guie´ et al., 2007) were reported. The expression of antioxidant related gene such as catalase, superoxide dismutase, ascorbate peroxidase and glutathione reductase were also observed during ripening of tomato and mango fruits (Jimeneze et al., 2002; Vasanthaiah et al., 2006).

CAT belongs to most important enzymes that can control levels of hydrogen peroxide. The K_m of CAT is relatively high, suggesting that the enzyme would be more active in the presence of high concentrations of H_2O_2. Lemoine et al. (2010) found no significant differences of CAT activity immediately after the combined treatment between control and treated broccoli. However, after 2 and 5 days of storage, a significant increase in the activity of controls was observed, which diminished after 7 days of storage. The activation of CAT activity in non-treated broccoli suggests a high accumulation of H_2O_2 that probably leads to an increase in catalase synthesis necessary to detoxify excess of H_2O_2. Instead, in treated samples, the increase in CAT activity was detected after 7 days of storage, indicating a delay in the accumulation of H_2O_2, which in turn suggest lower tissue damage due mainly to a delay in the senescence process.

Generally, plants show a rapid response to increased ROS produced by abiotic stresses like changes in storage temperature (Rogiers et al., 1998). These changes lead to an enhancement in cellular antioxidants that induce an increase in the activity of natural antioxidants and antioxidant enzymes such as SOD, CAT, GR and APX (Bowler et al., 1992).

The affinities of CAT (μM range) and other peroxidases such as ascorbate peroxidase (mM range) for hydrogen peroxide are very different which belong to two classes of hydrogen peroxide scavenging enzymes. Thus, although CAT is involved in the removal of excess reactive oxygen species (ROS) during stress conditions in plants, ascorbate peroxidase, which has been reported to be one of the most important antioxidant enzymes in defense against low temperature injury, appears to be responsible for the modulation of ROS for signaling (Mittlet, 2002).

Preconditioning treatments of fruit with hot water may induce chilling tolerance by modulating antioxidant systems that would prevent the accumulation of AOS (Martinez-Tellez and Lafuente, 1997; Sala and Lafuente, 2000). Hot water treatment can elevate CAT activity with decrease CI during storage. Generally, activity of antioxidant enzymes increased when the oxidant level increased with environmental stress (Ren et al., 1999). Therefore, the higher activity POX in peel

may be linked to the higher cell damage as a response to stress (Li, 2003). However, increase of CAT can reduce levels of oxidative damage.

Although there are many reports about effects of hot water treatment in antioxidant enzyme activity, the study concerning expression of antioxidant gene, especially *CAT* and *SOD* in banana, affected by hot water treatment is still absent.

In this study in, the activation of *CAT* expression in 'Hom Thong' banana can be observed immediately after hot water treatment until day 4 of storage and during fruit ripening at 25 °C. This result was related to the activity of CAT enzyme in a previous study (Nittaya Ummarat, 2005), which found the increase of CAT activity on day 3 and day 6 after storage. However, the decrease of CAT activity was found during the ripening where hot water treated fruits showed the higher activity than that of control fruits.

Increased CAT and POX activities in mango and banana during heat treatment followed by cooling at 8 ºC or 13 ºC, which indicated increased elimination of ROS, has been reported (Niranjana et al., 2009).

In grape berries, under chilling stress, the activities of CAT were significantly higher in heat treated fruit than those in control (Zhang et al., 2005). Furthermore, enhancement of CAT activity by heat treatment also had been reported in 'Fortune' mandarin fruits (Ghasemnezhad et al., 2008; Sala and Lafluente, 1999).

Zhang et al. (2005) found that the activities of CAT and POD in grape berries were reduced after heat pretreatment with 38 °C for 10 h, and chilling stress also induced a decrease in enzyme activity both in heat-pretreated and control grape berries. However, during subsequent chilling stress, the activities of CAT and POD in treated fruit were significantly higher than those in control.

Bassal and Hamahmy (2011) found that hot water dip treatment significantly increased CAT activity in 'Navel' and 'Valencia' fruit peel and juice under all storage conditions, compared with the control. Overall, the reduction in CI percentage was paralleled with higher CAT activity. The effects of heat treatment in elevating chilling tolerance maybe related to induction of CAT activity during heating and its tolerance during cold storage (Sala and Lafuente, 2000).

Superoxide dismutases (SOD) are metalloproteins catalyzing dismutation of the superoxide radical to molecular oxygen and H_2O_2, being the first line of defense from damages caused by oxygen radicals (Alscher et al., 2002). Several reports have shown that higher SOD activities can increase tolerance to different stress factors (Lee et al., 2001). Plants with an increased tolerance to heat stress have an elevated SOD activity (Almeselmani et al., 2006). Vicente et al. (2006) found that a longer

postharvest life of heat treated strawberry fruit was correlated with a higher SOD activity.

SOD is an important antioxidant enzyme, which scavenges reactive oxygen species thereby maintaining membranes of plant tissue (Lamb and Dixon, 1997). Jin et al. (2009) demonstrated that hot air (HA) treatment combined with methyl jasmonate (MJ) vapor treatment induced the activity of SOD. Furthermore, the maintenance of intact membranes of peach tissue found in HA and MJ treatments, as well as in their combination, might be another mechanism of alleviating internal browning.

According to *SOD* expression in this study, there was no correlation to our previous study that showed higher activity in hot water treatment only on day 0 (Nittaya Ummarat, 2005). During storage, the significant difference of SOD activity could not be found while the expressions of *SOD* in hot water treatment were higher than those of control on day 4 and day 6. This result was similar to Jimeneze et al. (2002), who found that changes of SOD activity were not related to changes in *SOD* gene expression. This incorrelation between enzyme activity and gene expression may be resulted from the degradation of mRNA during storage or post-translation process.

Ghasemnezhad et al. (2008) found an increase in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities after hot water treatments in mandarins. However, heat treatment followed by cooling did not enhance SOD activity in banana and mango fruits (Niranjana et al., 2009). This may be due to heat-induced ethylene in association with low temperature, maybe responsible for the lowered the SOD activity. These results are in corroboration with the study of Ben-Amor et al. (1999), who observed the effect of ethylene on reducing SOD activity during low temperature storage.

It has been found that heat treatments induced an increase in the activity of SOD, CAT and enzymes in ascorbate-glutathione cycle. HSPs have been proposed as synergistic factor acting with these enzymes or otherwise they might be related to the induction of these enzymes (Wahid et al., 2007). These antioxidants can help in controlling oxidative reactions caused by ROS in living tissues (Cisneros-Zevallos, 2003).

Maria et al. (2010) found that, immediately after heat treatments, an increase in SOD activity was observed and remained higher in treated samples after 5 days of storage. At the end of storage, SOD activity decreased in treated samples, while in control samples the SOD activity remained constant during storage. They suggest that the H_2O_2 generated by SOD may affect on accumulation of CAT and APX activities towards the end of the storage. Furthermore, the combined treatment

diminished POX activity, which suggests a minor role of this enzyme in detoxification of H_2O_2 in comparison to CAT and APX.

In present study, we found the lower *CAT* expression in hot water treated banana after storage at low temperature for 8 days. However, on day 10, day 14 and day 16, a higher *CAT* expression in treated fruit had been observed. In control fruits, *CAT* expressions were higher than those in hot water treated fruit on day 8 and 12, and then decreased to a lower level than those in hot water treated fruit during fruit ripening. This result was not related to CAT enzyme activity which increased on day 6 and then decreased throughout storage period in both treatments (Nittaya Ummarat, 2005).

The expression of *SOD* in hot water treatment were higher than those of control after removal from 14 °C on day 8, and during storage at 25 °C on day 10 and day 12. Compared to SOD enzyme activity, the increase of activity in hot water treatment was found on day 6 during storage at 14 °C and tended to decrease during storage at 25 °C. Higher SOD activities in hot water treatment were found on day 10 and day 14, while the higher *SOD* expressions in hot water treatment were shown on day 10 and day 12. Sapitnitskaya et al. (2006) reported that hot water treatment plus pre-storage at low temperature can activate *SOD* expression during chilling stress. That may be an effect of hot water treatment and low temperature storage on enhancement of stress tolerance in fruit.

Zhang et al. (2005) found that heat pretreatment induced a decrease in SOD activity after treatment, compared to the control. However, after chilling stress for 6 h, SOD activity increased in heat-pretreated berries and was higher than that in control over the following 72 h.

Regarding to *HSPs*, heat pre-treatment in grape berry induced an increase in the synthesis of *Hsp70* genes prior to the increase in activities of CAT, SOD and POD. When the decreases of *Hsp70* expression were observed, after chilling stress for 72 h, the lower activities of CAT and POD were also detected. This demonstrated that *Hsp70* might be related to the gene expression of antioxidant enzymes, and regulation of chilling tolerance (Zhang et al., 2005). Sapitnitskaya et al. (2006) reported that hot water treatments induced the expressions of *HSP*s, but down regulated *SOD* in grapefruit.

Heat treatment can affect on heat shock proteins (HSPs) and some oxygen radical scavenging genes such as *SOD, POX* and *CAT* (Vicente et al., 2006). Together with activation of other antioxidants such as polyphenolics, ascorbic acid and carotenoids, hot water treatment has a potential to decrease chilling injury symptom during low temperature storage, delay fruit ripening and reduce fugal attack during postharvest storage (Djioua et al., 2009; Talcott et al., 2005; Vicente et al., 2002).

Taken together, hot water treatment tended to affect some physiological changes, including antioxidant compounds and antioxidant encoding genes such as *CAT* and *SOD*. Enhancement by a minimal stress during hot water treatment before storage may activate a signal molecule as H_2O_2 to trigger antioxidant system in fruit in later period. As a result, antioxidant compounds such as, CAT and SOD could be elevated to decrease oxidative stress during storage. Moreover, the increase in active compound such as carotenoids, phenolics and flavonoids may suggest lower oxidative levels in fruit during storage and ripening, which might contribute to the delay ripening was shown in hot water treated fruits. This result supports the beneficial effects of hot water treatment to maintain quality and prolong shelf-life of banana fruits.

CHAPTER V

CONCLUSION

1. Effects of hot water treatment during storage at 25 °C

1.1 Effects on fruit quality

Ripening of banana fruit occurred on day 8 and up to day 10 of storage at 25 °C. According to L value, hue value and pulp firmness, hot water treatment showed a delay in ripening during day 8 to day 10.

1.2 Effects on oxidative stress level

The levels of H_2O_2 reduced in hot water treated banana during storage and ripening, although there was a tendency toward an increase of H_2O_2 in hot water treatment after treating on day 0 and day 2. Regarding MDA content, which reflects the level of lipid peroxidation, it was found that hot water treatment can also reduce MDA content in banana peel throughout storage time. As for electrolyte leakage, hot water treatment had a minor effect on an increase in electrolyte leakage after treating and during the early period of storage. However, the levels of electrolyte leakage of hot water treated fruits were lower than those of control.

1.3 Effects on antioxidants

Regarding antioxidant compounds, hot water treatment showed a small effect on AA contents at earlier periods of storage, while it tended to activate caroteniods and GSH contents during ripening. As a result, the ratio of GSH/GSSG, which indicates the oxidative stress level in cells, was higher in hot water treated fruits during become to ripe.

According to phenolics, flavonoids and DPPH radical scavenging activity, it was found that most of the active compounds were presented in free extracts. Hot water treatment tended to affect phenolics at the beginning of storage, but it had no significant effect during fruit ripening. As well as flavonoids, they increased in hot water treated fruit before ripening. DPPH radical scavenging activity, which indicates antioxidant capacity, decreased during storage; however, hot water treated fruits showed higher levels than those of control fruits.

1.4 Effects on *CAT* and *SOD* expression

Hot water treatment can activate *CAT* after treatment (0 h and 1 h), during early period of storage and also when fruit ripening. Hot water treatment shows no effect on *SOD* expression after treatment. The activation of *SOD* can be observed on day 4 and day 6 of storage, and then it was dropped during fruit ripening. Therefore, *CAT* seems to be an important gene that was induced by hot water treatment during storage at 25 °C.

According to the results, significantly beneficial effects of hot water treatment found in *Experiment 1* can be concluded in Table 5.1

Table 5.1 Beneficial effects of hot water treatment found in *Experiment 1.*

	Initial phase		Pre-ripening phase		Ripening phase	
Patameter:	Day0	Day2	Day4	Day6	Day8	Day10
L value	-	-	-	-	-	✓
Hue value	-	-	-	-	✓	✓
Pulp Firmness	-	-	-	-	✓	✓
H_2O_2	-	-	✓	-	✓	✓
MDA	-	✓	-	-	✓	-
Electrolyte leakage	-	-	-	-	✓	-
Carotenoids	-	-	-	-	✓	-
Ascorbic acid	-	✓	-	-	-	-
GSH	-	-	✓	-	✓	-
GSSG	-	-	-	-	-	-
GSH:GSSG	-	-	✓	-	✓	-
Bound phenolics	-	-	-	-	-	-
Free phenolics	-	-	✓	✓	-	-
Bound flavonoids	-	-	-	-	-	-
Free flavonoids	-	-	✓	✓	-	-
Bound antioxidant activity	-	-	-	-	-	-
Free antioxidant activity	-	-	-	-	-	-
CAT expression	✓	✓	✓	-	✓	✓
SOD expression	-	-	✓	✓	-	-

Where,

Initial phase: Day 0 to Day 2

Pre-ripening phase: Day 4 to Day 6

Ripening phase: Day 8 to Day 10

2. Effects of hot water treatment during storage at 14 °C for 8 days, followed by storage at 25 °C

2.1 Effects on fruit quality

For banana fruit stored at low temperature before being tranferred to 25 °C, ripening was delayed in both control and hot water treatment. Ripening could be observed on day 14 up to day 16 of storage, which delayed ripening was found in hot water treated fruits, as indicated by L value, hue value and pulp firmness.

2.2 Effects on oxidative stress level

H_2O_2 contents in hot water treated fruits were slightly increased before fruit ripening, while control fruits showed a sharply increase. However, MDA contents and electrolyte leakage in hot water treated fruit tended to be higher than those in control after being removed from 14 °C.

2.3 Effects on antioxidants

Hot water treatment had a minor effect on caroteniods, AA and glutathione contents. The increase of free phenolics and flavonoids in hot water treated fruits were higher than those of control during storage, although there was no significant difference during fruit ripening. Moreover, hot water treatment had no effect on antioxidant capacity throughout storage.

2.4 Effects on *CAT* and *SOD* expressions

CAT expression in hot water treatment was reduced after being removed from 14 °C, and then increased after 2 day of storage at 25 °C. When fruit began to ripen, *CAT* in hot water treated bananas were higher than in control fruits. *SOD* expression was induced when fruits were removed fruits from 14 °C and continuously exhibited higher expression in hot water treated fruits up to day 12 of storage. However, lower *SOD* expression was found in hot water treatment when fruit began to ripen.

It can conclude that *SOD* could be further activated through hot water treatment when fruits were stored at low temperature (14 °C) before being transferred to ripen at 25 °C and this effect can be found up to 4 days at 25 °C.

While during fruit began to ripen, *CAT* tended to show higher induction than *SOD.*

According to the results, significantly beneficial effects of hot water treatment found in *Experiment 1* can be concluded in Table 5.1

Table 5.2 Beneficial effects of hot water treatment found in *Experiment 2.*

	Initial phase		Pre-ripening phase		Ripening phase	
Patameter:	Day0	Day8	Day10	Day12	Day14	Day16
L value	-	-	-	-	-	✓
Hue value	-	-	-	-	-	✓
Pulp Firmness	-	-	✓	✓	✓	✓
H_2O_2	-	-	-	✓	-	✓
MDA	-	-	-	-	-	-
Electrolyte leakage	-	-	-	-	-	✓
Carotenoids	-	-	-	-	-	✓
Ascorbic acid	-	-	✓	✓	-	-
GSH	-	✓	✓	-	✓	-
GSSG	-	-	-	-	-	-
GSH:GSSG	-	-	✓	-	-	-
Bound phenolics	-	-	-	-	-	-
Free phenolics	-	✓	-	✓	-	-
Bound flavonoids	-	-	-	-	-	-
Free flavonoids	-	✓	-	✓	-	-
Bound antioxidant activity	-	-	-	-	-	-
Free antioxidant activity	-	-	-	-	-	-
CAT expression	✓	-	✓	-	✓	✓
SOD expression	-	✓	✓	✓	-	-

According to the results in *Experiment 1* and *Experiment 2*, we can suggest a model for an induction of antioxidant system in 'Hom Thong' banana fruit by hot water treatment, as shown in Figure 5.1. After banana fruits were treated by hot water treatment, in initial phase, electrolyte leakage increased and also affected on membrane lipid peroxidation, as indicated by MDA. Moreover, *CAT* was immediately induced after hot water treatment. In pre-ripening phase, *SOD* expressions were activated by hot water treatment. An increase in H_2O_2 was observed, which may be resulted from SOD enzyme function that releases H_2O_2 as a by-product. Also, the increases of electrolyte leakage in initial phase may affect in H_2O_2 accumulation. Furthermore, some antioxidant compounds were higher in hot water treated fruits, compared with

control fruits. As a consequence, *CAT* expressions were induced in ripening phase, while H_2O_2 content, electrolyte leakage and MDA were decreased. Finally, the level oxidative stress in hot water treated fruit should be lower than that in control fruit. This mechanism may be involved in the induction of hot water treatment that resulted in antioxidant system of 'Hom Thong' banana fruit during storage.

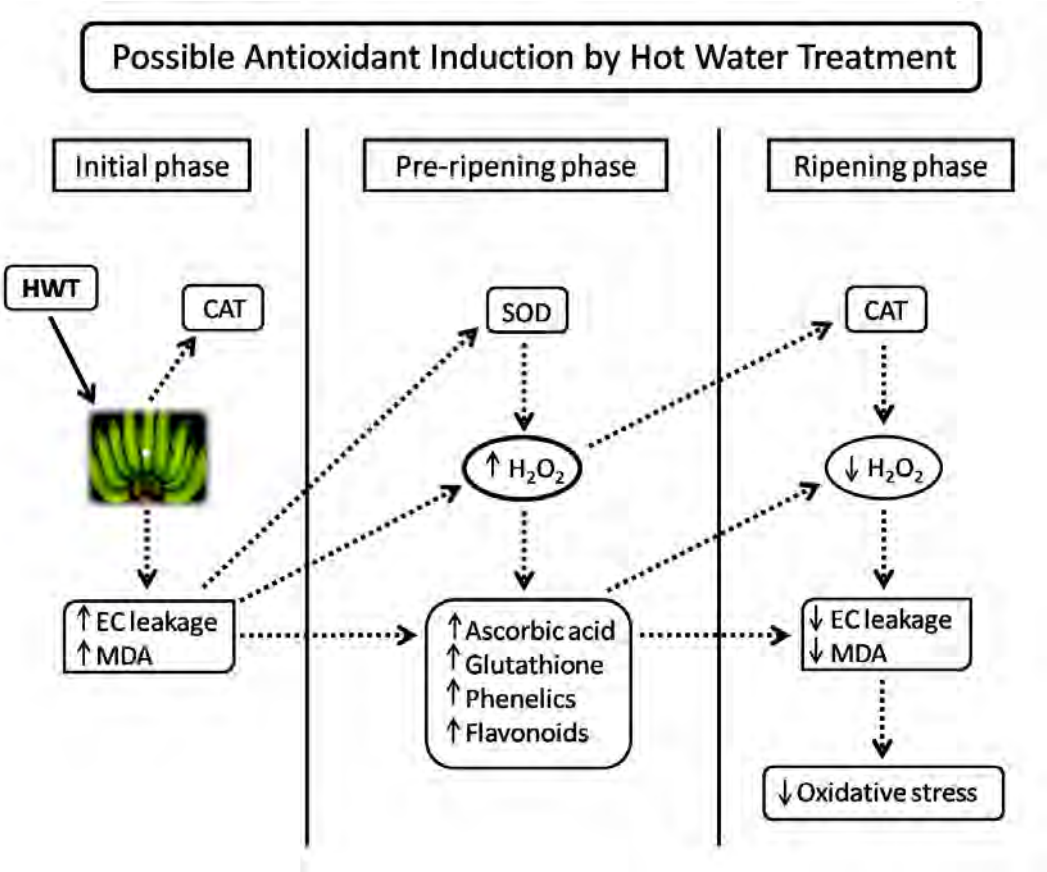


Figure 5.1 Possible induction of antioxidant system by hot water treatment in 'Hom Thong' banana fruit during storage.

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APPENDICES

APPENDIX A

Chemical solution

1. Electrophoresis

1.1. 5x TBE:

Tris base	54 g
Boric acid	27.5 g
EDTA pH 8.0	20 ml (0.5 M)

1.2 DNA loading dye and RNA loading dye for agarose gel:

Gltcerol in water	30% (v/v)
Bromophenol blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)

2. *DNase* treatment and reverse transcription

2.1. *DNase*I Treatment

Ten micrograms of total RNA sample were added to the *Dnase*I treatment mixture (1xDNase buffer and 1 unit of RQ1 DNase). The reaction was incubated at 37°C for an hour. After incubation, the mixture was added 100 µl of 40 mM Tris-HCl (pH 8.0) and one volume of phenol: chloroform: isoamylalcohol (25:24:1), then centrifuged at 12,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by the addition of 0.1 volumes of 3M NaOAc (pH 5.2) and 0.6 volumes of cooled isopropanol and stored at -20°C for 30 minutes. The mixture was centrifuged, the pellet was washed with 80% ethanol and briefly air-dried at room temperature. The DNA-free RNA pellet was resuspended in 10 µl DEPC-treated water.

2.2. Reverse Transcription

Two microgram of the *DNase*-treated RNA was added to the reverse transcription mixture (1x M-MLV Reverse Transcriptase buffer, 100 ng of oligo $(dT)_{15}$ primer). The reaction was incubated at 70 °C for 5 minutes, then quickly cooled on ice. After that, 100 ng of the dNTP mix and 200 units of M-MLV Reverse Transcriptase were added to the reaction mixture for the 20 µlfinal volume. The reaction mixture were then incubated at 42°C for an hour. The first strand cDNA was stored at - 20°C for further use of Real time PCR.

3. Real-time polymerase chain reaction

The specific primer sequences for each gene were designed. The parallel amplication of *EF-1α*, the housekeeping control gene in banana, was used to normalize gene expression. Real-time PCR technique was used for quantitatively determine the expression profile of the *CAT* and *SOD*. The quantitative real-time PCR was conducted on CFX96 Real-time system (Bio-Rad, USA). At least 3 independent real-time PCR reactions were performed on the same cDNA preparation. Each reaction contained 10 µl of 2x Prime Q Master Mix Reagent (with SYBR Green), 1 µl of cDNA samples, and 0.5 ul of 10 µM gene-specific primers in a final volume of 20 µl. The thermal cycle used was as follows: (1) 95°C for 10 sec, (2) 95°C for 30 sec, (3) 62°C for 30 sec, (4) 72 C for 30 sec + plate read, (5) go to (2) for 39 more times, (6) 95 C for 10 sec, and (7) melt curve 65 to 95 C for 5 sec + plate read.

4.1 Calculation of relative gene expression level

The level of gene expression was determined in comparison with the housekeeping gene (reference gene) expression in reference to the expression on day0 of the treatment (control). For the detection in various rice organs, the gene expression was calculated in reference to the expression level of young leaf blade tissues. The relative expression ratio of target gene was calculated based on PCR efficiency (E) and the CP deviations. CP was defined as the point at which the florescence rises appreciably above the background fluorescence (Pfaffl, 2001).

$$\text{Relative gene expression level} = \frac{(E_{target})^{\Delta CTtarget}}{(E_{ref})^{\Delta CTref}}$$

E_{target} = $10^{(-1/slope)}$ of *CAT* or *SOD*

E_{ref} = $10^{(-1/slope)}$ of *EF-1α*

ΔCT_{target} = $CT_{control\ at\ 0h} - CT_{sample\ at\ any\ time\ point}$ of *CAT* or *SOD*

ΔCT_{ref} = $CT_{control\ at\ 0h} - CT_{sample\ at\ any\ time\ point}$ of *EF-1α*

APPENDIX B

Table B.1 Physiological and biochemical changes of banana during storage at 25 °C

Physical/Biochemical changes:	Treatment	Day0	Day2	Day4	Day6	Day8	Day10
L-value	Control	56.88±0.67	54.82±0.43	56.81±0.99	58.50±1.18	65.59±1.55	72.17±0.47
	HWT	53.70±0.51	54.25±0.76	57.91±1.33	59.59±1.00	62.10±1.41	63.07±1.14*
Hue-value	Control	109.59±0.23	108.92±0.15	106.46±1.36	104.86±1.69	91.46±2.75	92.13±0.46
	HWT	109.74±0.22	108.70±0.25	104.00±1.72	105.71±0.99	103.46±2.98*	99.49±3.29*
Pulp firmness (N)	Control	7.73±0.03	7.58±0.05	7.21±0.14	6.89±0.15	5.15±0.36	4.44±0.22
	HWT	7.76±0.03	7.70±0.03	6.96±0.21	7.03±0.11	6.70±0.15*	5.31±0.26*
Carotenoids (μmol/g FW)	Control	28.14±1.20	32.21±1.07	28.16±1.76	26.05±0.95	20.77±1.44	63.50±3.28
	HWT	27.33±1.86	33.06±3.42	27.88±1.24	25.72±0.77	25.02±0.12*	73.21±6.60
AA (mg/g FW)	Control	18.36±0.84	14.12±0.43	14.17±0.52	13.04±0.49	21.18±1.27	22.55±2.48
	HWT	18.05±0.87	18.21±1.05*	15.35±0.36	14.10±0.40	18.88±1.99	21.94±2.83
GSH (μmol/g FW)	Control	0.111±0.012	0.072±0.012	0.125±0.008	0.051±0.007	0.037±0.003	0.039±0.003
	HWT	0.129±0.014	0.109±0.015	0.163±0.001*	0.052±0.005	0.236±0.017*	0.044±0.007
GSSH (μmol/g FW)	Control	0.218±0.064	0.289±0.063	0.171±0.055	0.204±0.049	0.112±0.008	0.120±0.010
	HWT	0.187±0.060	0.108±0.035*	0.026±0.005*	0.155±0.051	0.014±0.004*	0.218±0.039*
GSH:GSSH	Control	1.181±0.657	0.569±0.393	1.778±0.823	0.350±0.129	0.330±0.001	0.328±0.002
	HWT	1.387±0.658	2.276±1.032	7.553±1.763*	0.627±0.260	16.515±2.5911*	0.319±0.165

* indicates the significant difference between control and hot water treatment ($P< 0.05$)

Table B.1 (*cont.*) Physiological and biochemical changes of banana during storage at 25 °C

Physical/Biochemical changes:	Treatment	Day0	Day2	Day4	Day6	Day8	Day10
H_2O_2 (μmol/g FW)	Control	7.84±0.63	6.77±0.76	8.57±0.22	9.01±0.78	9.34±0.67	10.29±0.66
	HWT	8.27±1.21	7.65±0.84	7.73±0.28*	7.24±0.41	7.44±0.41*	7.48±0.95*
MDA (μmol/g FW)	Control	3.56±0.53	4.30±0.29	3.84±0.35	4.16±0.53	3.54±0.40	3.70±0.42
	HWT	3.19±0.36	3.49±0.03*	3.16±0.26	3.24±0.31	2.48±0.01*	3.43±0.23
EC leakage (%)	CT	7.20±0.96	4.85±1.12	27.41±7.29	3.58±1.46	9.78±1.29	17.49±4.90
	HWT	10.13±1.45	7.37±1.09	37.03±7.98	1.03±0.52	3.94±1.05*	13.31±5.23
Bound phenolics (mg/g FW)	CT	0.018±0.003	0.018±0.005	0.021±0.006	0.016±0.006	0.023±0.008	0.018±0.007
	HWT	0.015±0.005	0.017±0.004	0.023±0.008	0.017±0.004	0.020±0.007	0.018±0.006
Free phenolics (mg/g FW)	CT	1.043±0.063	1.155±0.080	1.099±0.128	0.952±0.058	1.550±0.062	1.242±0.102
	HWT	1.283±0.278	1.268±0.094	1.896±0.275*	1.139±0.024*	1.348±0.146	1.393±0.081
Bound flavonoids (mg/g FW)	CT	0.022±0.002	0.024±0.002	0.030±0.004	0.024±0.004	0.039±0.008	0.035±0.003
	HWT	0.027±0.004	0.022±0.002	0.034±0.003	0.029±0.002	0.032±0.002	0.031±0.002
Free flavonoids (mg/g FW)	CT	2.682±0.099	2.733±0.205	3.490±0.063	2.437±0.074	3.700±0.109	3.143±0.105
	HWT	2.870±0.150	2.834±0.392	4.277±0.209*	3.413±0.133*	3.479±0.091	3.199±0.161
Bound DPPH radical scavenging (%)	CT	45.94±8.29	58.71±2.05	58.55±5.41	51.06±3.86	60.40±7.47	51.17±7.54
	HWT	66.26±5.37	61.26±6.94	57.16±4.49	61.46±8.16	54.66±5.07	59.05±5.82
Free DPPH radical scavenging (%)	CT	72.65±1.00	71.32±1.70	73.45±0.47	71.85±0.22	69.16±0.66	70.29±0.46
	HWT	71.95±0.77	73.55±1.19	73.09±0.48	72.46±0.64	70.54±0.76	70.64±0.46

* indicates the significant difference between control and hot water treatment ($P< 0.05$)

Table B.2 Physiological and biochemical changes of banana during storage at 14 °C for 8 days, followed by storage at 25 °C

Physical/Biochemical changes:	Treatment	Day0	Day8	Day10	Day12	Day14	Day16
L-value	Control	55.02±0.41	52.87±0.39	54.54±0.29	56.26±0.37	58.07±0.59	64.65±0.63
	HWT	54.57±0.38	54.26±0.0.49*	55.92±0.25*	55.65±0.30	58.20±0.40	61.93±0.55*
Hue-value	Control	119.73±0.14	119.91±0.13	118.82±0.23	118.74±0.49	114.28±1.67	105.06±1.87
	HWT	120.23±0.11	118.98±0.14*	117.96±0.28*	117.75±0.26	115.27±1.13	110.40±1.67*
Firmness	Control	7.52±0.10	7.40±0.12	7.35±0.05	7.37±0.06	6.64±0.20	5.61±0.22
	HWT	7.69±0.03	7.50±0.10	7.50±0.05*	7.59±0.04*	7.30±0.10*	6.54±0.21*
Carotenoids (μmol/g FW)	Control	28.72±1.90	28.39±0.36	22.31±0.97	30.62±0.73	67.49±16.67	91.69±10.18
	HWT	29.38±1.06	28.30±1.27	26.43±1.93	34.36±0.42	71.82±32.17	118.85±5.45*
AA (mg/g FW)	Control	20.04±0.98	25.55±1.70	23.47±0.99	13.10±0.81	15.73±1.09	14.95±0.83
	HWT	19.96±1.15	28.33±3.06	27.32±0.97*	17.68±1.27*	15.92±0.62	17.63±1.51
GSH (μmol/g FW)	Control	0.065±0.013	0.071±0.011	0.047±0.011	0.063±0.002	0.074±0.005	0.114±0.038
	HWT	0.076±0.012	0.124±0.006*	0.116±0.011*	0.071±0.014	0.099±0.004*	0.119±0.014
GSSH (μmol/g FW)	Control	0.345±0.058	0.323±0.089	0.249±0.052	0.311±0.063	0.283±0.112	0.157±0.077
	HWT	0.396±0.077	0.451±0.110	0.115±0.051	0.367±0.082	0.256±0.119	0.139±0.071
GSH:GSSH	Control	0.233±0.077	0.492±0.259	0.288±0.122	0.237±0.041	0.594±0.249	1.757±0.867
	HWT	0.215±0.039	0.341±0.074	1.953±0.667*	0.322±0.171	1.355±0.604	1.618±0.510

* indicates the significant difference between control and hot water treatment ($P< 0.05$)

Table B.2 (cont.) Physiological and biochemical changes of banana during storage at 14 °C for 8 days, followed by storage at 25 °C

Physical/Biochemical changes:	Treatment	Day0	Day8	Day10	Day12	Day14	Day16
H_2O_2 (μmol/g FW)	Control	8.06±0.80	8.62±1.58	12.32±2.04	24.70±1.45	7.59±0.77	9.68±0.37
	HWT	8.39±0.80	5.70±0.24	10.49±0.70	12.46±2.44*	7.51±0.32	6.54±0.92*
MDA (μmol/g FW)	Control	3.55±0.52	4.97±0.26	2.47±0.02	5.17±0.59	5.82±0.33	3.53±0.40
	HWT	3.57±0.21	5.82±0.48	3.37±0.10*	4.83±0.19	6.05±0.65	4.73±0.38
EC leakage (%)	CT	7.20±0.96	6.81±1.14	3.91±0.97	5.20±0.65	4.35±1.66	5.63±0.85
	HWT	10.130±1.45	10.47±1.52	5.93±0.97	5.30±0.50	2.87±0.51	3.29±0.15*
Bound phenolics (mg/g FW)	CT	0.018±0.003	0.018±0.004	0.022±0.003	0.017±0.005	0.023±0.005	0.018±0.007
	HWT	0.015±0.005	0.017±0.004	0.015±0.005	0.017±0.005	0.022±0.006	0.016±0.007
Free phenolics (mg/g FW)	CT	1.043±0.063	1.279±0.042	1.400±0.089	1.239±0.036	1.486±0.078	1.618±0.044
	HWT	1.283±0.278	1.591±0.056*	1.492±0.041	1.668±0.071*	1.556±0.058	1.530±0.082
Bound flavonoids (mg/g FW)	CT	0.022±0.002	0.024±0.003	0.025±0.002	0.024±0.002	0.025±0.004	0.035±0.004
	HWT	0.027±0.004	0.026±0.003	0.032±0.003	0.028±0.003	0.034±0.004	0.034±0.002
Free flavonoids (mg/g FW)	CT	2.682±0.099	2.605±0.062	3.374±0.142	2.754±0.100	3.626±0.010	3.605±0.205
	HWT	2.870±0.150	3.558±0.056*	3.365±0.216	3.483±0.178*	3.360±0.114	3.757±0.173
Bound DPPH radical scavenging (%)	CT	45.94±8.29	47.06±12.06	53.47±9.45	57.79±5.02	49.05±10.97	47.37±10.93
	HWT	66.26±5.37	55.13±6.58	54.74±3.81	50.23±8.39	49.89±9.13	52.48±9.54
Free DPPH radical scavenging (%)	CT	72.65±1.00	67.35±0.45	68.71±0.39	66.60±0.22	66.01±0.36	65.06±0.45
	HWT	71.95±0.77	67.96±0.52	68.76±0.64	67.07±0.33	66.35±0.62	66.42±0.75

* indicates the significant difference between control and hot water treatment ($P< 0.05$)

Table B.3 Relative gene expressions of banana after hot water treatment

Gene	Treatment	0h	1h	3h
CAT	CT	1.000±0.000	4.178±0.334	7.340±0.320
	HWT	8.925±1.438*	4.750±0.263	3.069±0.289*
SOD	CT	1.00±0.000	0.002±0.000	1.677±0.046
	HWT	0.0213±0.002*	0.007±0.001	0.569±0.010*

* indicates the significant difference between control and hot water treatment ($P< 0.05$)

Table B.4 Relative gene expressions of banana during storage at 25 °C

Gene	Treatment	Day0	Day2	Day4	Day6	Day8	Day10
CAT	CT	1.000±0.000	0.922±0.029	0.525±0.043	7.791±0.351	1.341±0.079	2.947±0.076
	HWT	8.925±1.438*	5.713±0.048*	8.201±0.055*	0.516±0.028*	3.429±0.333*	6.399±0.879*
SOD	CT	1.00±0.000	0.232±0.016	1.062±0.065	0.300±0.028	0.843±0.091	1.183±0.074
	HWT	0.0213±0.002*	0.214±0.030	4.938±0.253*	1.037±0.015*	0.099±0.007*	0.196±0.024*

* indicates the significant difference between control and hot water treatment ($P< 0.05$)

Table B.5 Relative gene expressions of banana during storage at 14 °C for days, followed by storage at 25 °C

Gene	Treatment	Day0	Day8	Day10	Day12	Day14	Day16
CAT	CT	1.000±0.000	16.277±1.115	2.062±0.101	22.058±0.856	1.029±0.114	0.553±0.035
	HWT	8.925±1.438*	8.594±0.677*	6.332±0.063*	4.679±0.660*	4.684±0.211*	0.985±0.086*
SOD	CT	1.00±0.000	0.379±0.022	0.531±0.036	0.696±0.017	3.680±0.284	1.081±0.044
	HWT	0.021±0.002*	0.656±0.040*	0.937±0.070*	1.635±0.068*	0.459±0.027*	0.486±0.045*

Table B.6 Correlation between all parameters of control banana during storage at 25 °C

Parameters	Hue	Firmness	Carotenoids	MDA	GSH	GSSH	GSH:GSSH	H_2O_2	AA	EC leakage	Bound Phenolics	Bound flavonoids	Bound DPPH	Free phenolics	Free Flavonoids	Free DPPH	*CAT*	*SOD*
Hue	-	0.982**	-	-	-	0.865*	0.865*	-0.828*	-	-	-	-0.923*	-	-	-	0.834*	-	-
Firmness	0.982**	-	-	-	-	0.842*	0.842*	-0.863*	-	-	-	-0.873*	-	-	-	-	-	-
Carotenoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.845*
GSH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.894*	-	-
GSSH	0.865*	0.842*	-	-	-	-	1.000*	-0.914*	-	-	-	-0.869*	-	-	-	-	-	-
GSH:GSSH	0.865*	0.842*	-	-	-	1.000*	-	-0.914*	-	-	-	-0.869*	-	-	-	-	-	-
H_2O_2	-0.828*	-0.863*	-	-	-	-0.914*	-0.914*	-	-	-	-	-	-	-	-	-	-	-
AA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EC leakage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-Phenolics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.941**	-	-	-
B-flavonoids	-0.923**	-0.873*	-	-	-	-0.869*	-0.860*	-	-	-	-	-	-	0.866*	0.863*	-	-	-
B-DPPH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F-phenolics	-	-	-	-	-	-	-	-	-	-	-	0.866*	-	-	-	-	-	-
F-Flavonoids	-	-	-	-	-	-	-	-	-	-	0.941**	0.863*	-	-	-	-	-	-
F-DPPH	0.834*	-	-	-	0.894*	-	-	-	-	-	-	-	-	-0.817*	-	-	-	-
CAT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOD	-	-	-	-0.485*	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table B.7 Correlation between all parameters of hot water treated banana during storage at 25 °C

Parameters	Hue	Firmness	Carotenoids	MDA	GSH	GSSH	GSH:GSSH	H_2O_2	AA	EC leakage	Bound Phenolics	Bound flavonoids	Bound DPPH	Free phenolics	Free Flavonoids	Free DPPH	*CAT*	*SOD*
Hue	-	0.966**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Firmness	0.966*	-	-0.821*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carotenoids	-	-0.821*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MDA	-	-	-	-	-0.835*	-	-	-	-	-	-	-	-	-	-	-	-	-
GSH	-	-	-	-0.835*	-	-0.838*	-0.838*	-	-	-	-	-	-	-	-	-	-	-
GSSH	-	-	-	-	-0.838*	-	1.000*	-	-	-	-	-	-	-	-	-	-	-
GSH:GSSH	-	-	-	-	-0.838*	1.000**	-	-	-	-	-	-	-	-	-	-	-	-
H_2O_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.838*	-
AA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EC leakage	-	-	-	-	-	-	-	-	-	-	-	-	-	0.969*	-	-	-	0.881*
B-Phenolics	-	-	-	-	-	-	-	-	-	-	-	-	-0.824*	0.851*	0.916*	-	-	-
B-flavonoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.825*	-	-	-
B-DPPH	-	-	-	-	-	-	-	-	-	-	-0.824*	-	-	-	-	-	-	-
F-phenolics	-	-	-	-	-	-	-	-	-	0.969*	0.851*	-	-	-	-	-	-	0.876*
F-Flavonoids	-	-	-	-	-	-	-	-	-	-	0.916*	0.825*	-	-	-	-	-	0.891*
F-DPPH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CAT	-	-	-	-	-	-	-	0.838*	-	-	-	-	-	-	-	-	-	-
SOD	-	-	-	-	-	-	-	-	-	0.881*	-	-	-	0.876*	0.891*	-	-	-

* Correlation is significant at the 0.05 level (2-tailed)
** Correlation is significant at the 0.01 level (2-tailed)

Table B.8 Correlation between all parameters of control banana during storage at 14 °C for days, followed by storage at 25 °C

Parameters	Hue	Firmness	Carotenoids	MDA	GSH	GSSH	GSH:GSSH	H_2O_2	AA	EC leakage	Bound Phenolics	Bound flavonoids	Bound DPPH	Free phenolics	Free Flavonoids	Free DPPH	*CAT*	*SOD*
Hue	-	0.995*	-0.958**	-	-0.904**	0.889*	-0.963**	-	-	-	-	-0.955**	-	-0.813*	-	-	-	-
Firmness	0.995**	-	-0.973**	-	-0.901*	0.878*	-0.963**	-	-	-	-	-0.942**	-	-0.847**	-	-	-	-
Carotenoids	-0.958*	-0.973**	-	-	0.899*	-	0.899*	-	-	-	-	0.843*	-	-	-	-	-	-
MDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GSH	-0.904*	-0.901*	0.899*	-	-	-	0.943**	-	-	-	-	0.859*	-	-	-	-	-	-
GSSH	0.889*	0.878*	-	-	-	-	-0.866*	-	-	-	-	-0.947**	-	-0.884*	-	-	-	-
GSH:GSSH	-0.963**	-0.960**	0.899*	-	0.943**	-0.866*	-	-	-	-	-	0.971**	-	-	-	-	-	-
H_2O_2	-	-	-	-	-	-	-	-	-	-	-	-	-0.909*	-	-	-	-	-
AA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EC leakage	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-Phenolics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-flavonoids	-0.955**	-0.942**	0.843*	-	0.859*	-0.947*	0.971**	-	-	-	-	-	-	0.816*	-	-	-	-
B-DPPH	-	-	-	-	-	-	-	0.909*	-	-	-	-	-	-	-	-	-	-
F-phenolics	-0.813*	-0.847*	-	-	-	-0.884*	-	-	-	-	-	0.816*	-	-	0.874*	0.826*	-	-
F-Flavonoids	-	-	-	-	-	-	-	-	-	-	-	-	-	0.874*	-	-	-	-
F-DPPH	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.826*	-	-	-	-
CAT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* Correlation is significant at the 0.05 level (2-tailed)

** Correlation is significant at the 0.01 level (2-tailed)

Table B.9 Correlation between all parameters of hot water treated banana during storage at 14 °C for days, followed by storage at 25 °C

Parameters	Hue	Firmness	Carotenoids	MDA	GSH	GSSH	GSH:GSSH	H_2O_2	AA	EC leakage	Bound Phenolics	Bound flavonoids	Bound DPPH	Free phenolics	Free Flavonoids	Free DPPH	*CAT*	*SOD*
Hue	-	0.970**	-0.969**	-	-	-	-	-	-	-	-	-	-	-	-	-	0.941**	-
Firmness	0.970**	-	-0.958**	-	-	-	-	-	-	-	-	-	-	-	-	-	0.848*	-
Carotenoids	-0.969**	-0.958**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.867*	-
MDA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GSH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GSSH	-	-	-	-	-	-	-0.963**	-	-	-	-	-0.876*	-	-	-	-	-	-
GSH:GSSH	-	-	-	-	-	-0.963**	-	-	-	-	-	0.885*	-	-	-	-	-	-
H_2O_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EC leakage	-	-	-	-	-	-	-	-	-	-	-	-0.891*	-	-	-	-	0.885*	-
B-Phenolics	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-flavonoids	-	-	-	-	-	-0.876*	0.885*	-	-	-0.891*	-	-	-	-	-	-	-	-
B-DPPH	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.906*	-	0.963*	-	-
F-phenolics	-	-	-	-	-	-	-	-	-	-	-	-	-0.906*	-	-	-0.852*	-	-
F-Flavonoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-0.861*	-	-
F-DPPH	-	-	-	-	-	-	-	-	-	-	-	-	0.963**	-0.852*	-0.861*	-	-	-
CAT	0.941**	0.848*	-0.867*	-	-	-	-	-	-	0.885*	-	-	-	-	-	-	-	-
SOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

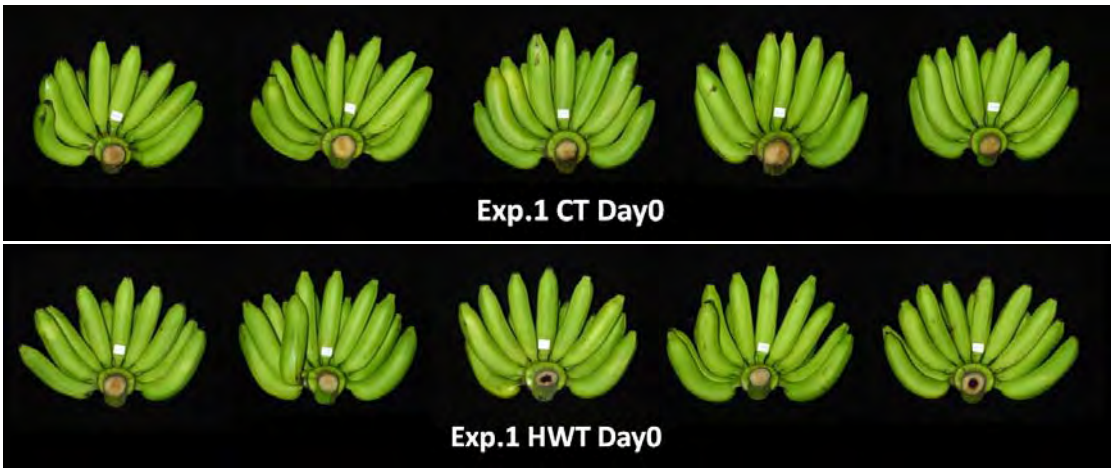
* Correlation is significant at the 0.05 level (2-tailed)

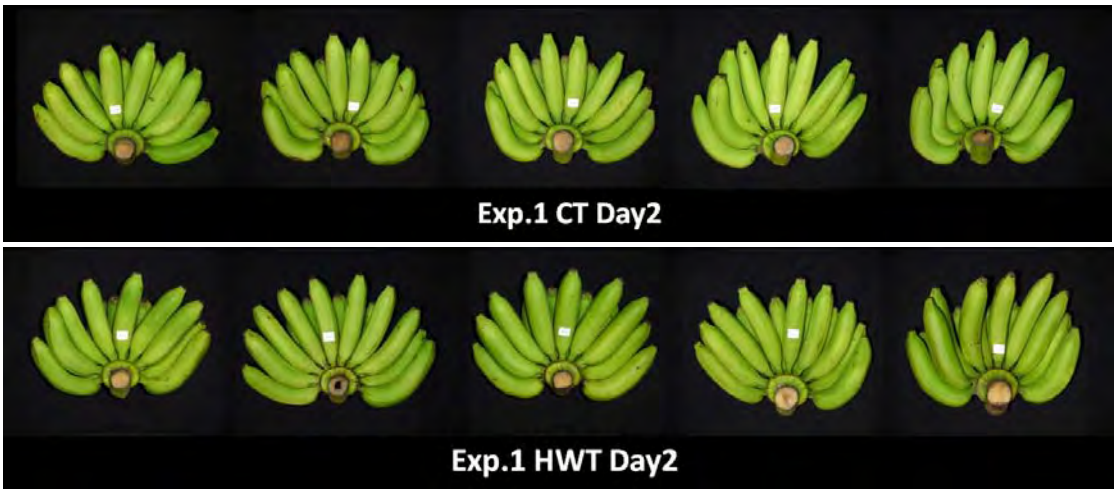
** Correlation is significant at the 0.01 level (2-tailed)

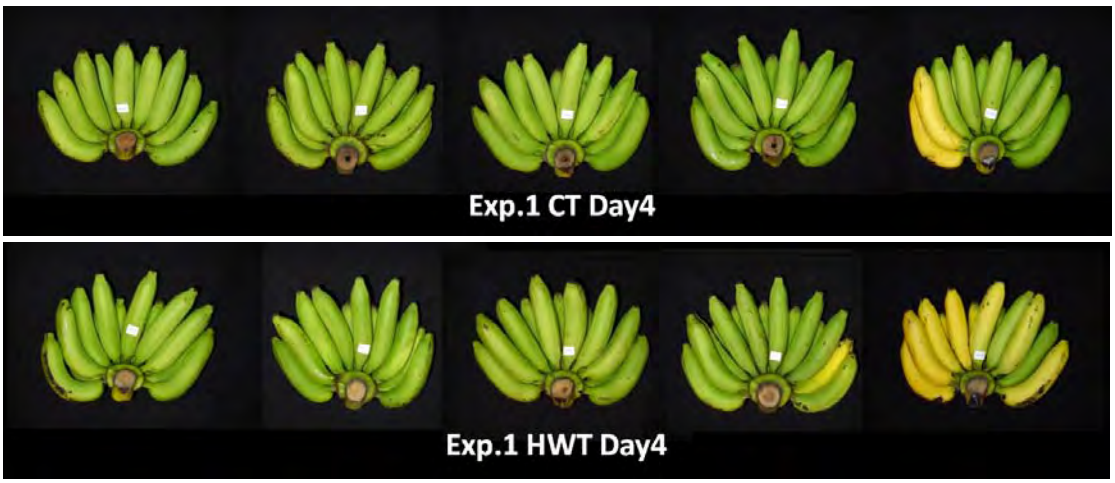
APPENDIX C

External appearance of banana fruits

1. Experiment 1: storage at 25 °C.








Exp.1 CT Day6


Exp.1 HWT Day6

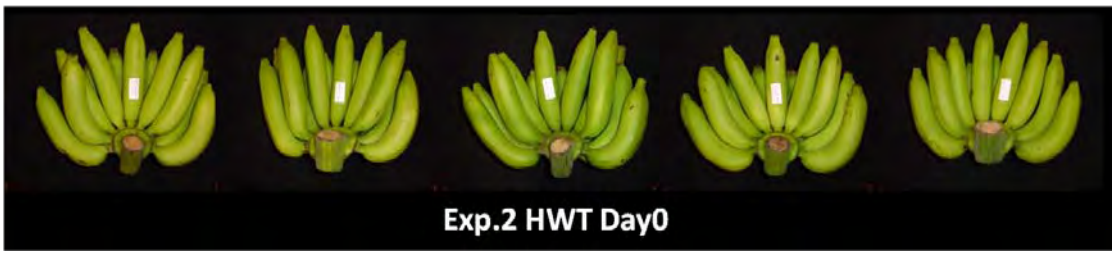

Exp.1 CT Day8

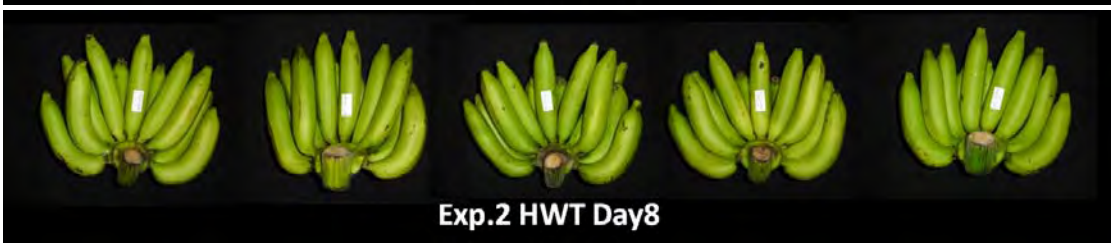

Exp.1 HWT Day8


Exp.1 CT Day10


Exp.1 HWT Day10

2. Experiment 2 : storage at 14 °C for 8 days, followed by storage at 25 °C.






Exp.2 CT Day12


Exp.2 HWT Day12


Exp.2 CT Day14


Exp.2 HWT Day14


Exp.2 CT Day16


Exp.2 HWT Day16

APPENDIX D

Gene cloning from *Musa acuminate* (AAA group, cv 'Blue field') banana peel

1. Elongation factor-1 alpha (*EF-1α*)

EF-1α was used as a reference gene for gene expression. Forward and reverse primers were designed according to EF-1α of *Musa acuminata* sequence from NCBI database (Accession no. DQ057979).

Forward primer: 5′ GCC ATG TCG ATT CTG GTA AA 3′
Reverse primer: 5′ GGA GCA TCA ATG VA GTG CAA 3′

Then, these primers were used to clone EF-1α from cDNA of 'Blue field' banana. The PCR conditions are:

1) 94 °C, 5 min
2) 94 °C, 30 sec
3) 55 °C, 30 sec
4) 72 °C, 2 min
5) Go to 2), 29 more times
6) 72 °C, 10 min
7) 4 °C, hold.

Products from PCR were cloned into pGEMT vector and transformed to *E. coli* (XL-1 Blue cells-PBARC) to amplify. After that all clones were sequenced and blast to data base to confirm similarity with *EF-1α* of *Musa sp*.

The sequences of *EF-1α* that were isolated are:

GCATGCTCCCGGCCGCCATGGCTGCCGCGGGAATTCGATT GCCATGTCGATTCTGGTAAA
TCGACAACAACTGGACATCTGATTTACAAACTTG**GGGGGATTGACAAGCGTGTTATCG**AGA
GGTTTGAGAAGGAAGCTGCTGAGATGAACAAGAGGTCATTCAAGTATGCGTGGGTGCTTGA
CAAGCTGA**AGGCTGAGCGTGAACGTGGT**ATCACTATCGATATCGCTCTGTGGAAGTTTGAG
ACCACAAAGTACTATTGCACTGTCATTGATGCTCCAATCACTAGTGAATTCGCGGCCGCCT
GCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAG
TGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC
CGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTA
ATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAAC
CTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTG
GGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGC
GGTATCAGCTCACTCAGAGGCGGTANTTT

Then, from these sequences, primer pairs for real time PCR were designed. According to the very close relationship between 'Blue field' and 'Hom Thong' banana, which are classified in 'AAA' groups. Primers for real time PCR in 'Hom Thong' banana in this study were designed by using the sequences from 'Blue field'. The primers are:

Forward primer: 5′ GGG GGA TTG ACA AGC GTG TTA TCG 3′
Reverse primer: 5′ ACC ACG TTC ACG CTC AGC CT 3′

2. SOD

SOD from 'Blue field' was cloned by using primers designed according to *SOD* from *Musa acuminata* (Acession no. AF510071). The primers are:

Forward primer: 5′ TCA CCA ACT ACA ACA ATG CCC 3′
Reverse primer: 5′ TGC ATG TTC CCA CAC ATC AA 3′

The sequences of *SOD* that were isolated are:

CGCATGCTCCCGGCCGCCATGGCGTGCCGCGGGAATTCGATTCAAGTTCAAT**GGTGGAGG**
TCATATCAACCACTCGATCTTCTGGAAAAATCTCAAGCCTGTGAATGAAGGTGGGGGTGA
GCCTCCACACAGCACACTTGGTTGGGCAATCGATACAGACTTTTGTTCTTTGGAGGCTCT
GGTGCAGAAAATGAAT**GCAGAAGGTGCTGCTTTGCAG**GGATCTGGATGGGTGTGGTTAGC
TCTGGATAAGGGAAGCAAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGG
GAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCT
TGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC
ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAC
TCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC
TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCG
CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTC
ACTCAAAGGCGGTAATACGGTTATTCACAGAATCAGGGGATAACGCAGGAAAGAACATGT
GAGCCAAAGGGCCAGCAAAAGGCCAGGA

According to these sequences, primer pairs for real time PCR were designed. The primers are:

Forward primer: 5′ GGT GGA GGT CAT ATC AAC CAC TCG 3′
Reverse primer: 5′ CTG CAA AGC AGC ACC TTC TGC 3′

3. CAT

CAT from 'Blue field' was cloned by using primers designed according to *CAT2* from *Musa acuminata* (Acession no. EU139298). The primers are:

Forward primer: 5′ ATC CTT ACA AGT TCC GTC CCT 3′
Reverse primer: 5′ TTG CTC TCA TGT TGA GGC GAT 3′

The sequences of *CAT* that were isolated are:

GCATGCTCCCGGCCGCCATGGCTGGCCGCGGGAATTCGATTTATGGAACGATGACCAGGC
CCTCACCGTGGGATCCAGAGCTTATCATTGCTCTCAGGACCCATCCTCCTTGAGGACTAC
CATCTGGTCGAAAAGATCGCTCACTTCGCCCGGGA**GCGCATACCGGAGCGCGTCGT**CCAT
GCCCGAGGCGCGAGCGCCAAGGGCTTCTTCGAGTGCACTCATGATGTCACTCACCTCACC
TGCGCCGACTTCCTCCGGGCGCCCGGCGTCCAGACGCCGATCATCCTCCGCTTCTCCACC
GTCATCCACGAGCGTGGCAGCCCTGACCACCATCAGAGACCCCCGCGGGTTCGCCGTCAA
GTTCTACACAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTC
CCAACGCGTCGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAA
TCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA
CGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAA
TTGAATCGGCCAACGCGCGGGGGAGNAA

According to these sequences, primer pairs for real time PCR were designed. The primers are:

Forward primer: 5′ GCG CAT ACC GGA GCG CGT CGT 3′
Reverse primer: 5′ GCT GCC ACG CTC GTG GAT GAC 3′

BIOGRAPHY

Miss Nittaya Ummarat was born on July 20, 1980 in Trang Province. She finished the secondary school from Princess Chulabhorn's College Trang in 1999. Then, she got the scholarship from the Development and Promoting Sciences and Technology Talents Project (DPST) to study in Bachelor's degree, majored in Biology, Faculty of Science, at Prince of Songkla University from 1999 to 2003. After that she continued on Master degree in the Department of Botany, Faculty of Science, Chulalongkorn University from 2003 to 2006. She has been supported by DPST to study and doing her research for the degree of Doctor of Philosophy in Biological Science program, Faculty of Science, Chulalongkorn University since 2006.