THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Doctor of Philosophy (Postharvest Technology)

DEGREE

Postharvest Technology — **FIELD**

Interdisciplinary Graduate Program — **PROGRAM**

TITLE: Effects of Calcium, Gibberellins and Abscisic Acid on Internal Browning of Pineapple

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THESIS

ROLE OF CALCIUM, GIBBERELLINS AND ABSCISIC ACID ON INTERNAL BROWNING OF PINEAPPLE

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A Thesis Submitted in Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy (Postharvest Technology)
Graduate School, Kasetsart University
2014

Issaya Pusittigul 2014: Effects of Calcium, Gibberellins and Abscisic Acid on Internal Browning of Pineapple. Doctor of Philosophy (Postharvest Technology), Major Field: Postharvest Technology, Interdisciplinary Graduate Program. Thesis Advisor: Professor Jingtair Siriphanich, Ph.D. 137 pages.

Internal browning (IB) of pineapples (*Ananas comosus* L. Merr) limits the storage life and transportation potential at low temperature. Queen (cvs. Phulae and Trad-see-thong) and Smooth Cayenne (cvs. Nanglae and Pattavia) pineapples were harvested from various planting locations in Thailand. Total calcium content was determined before storage and the remainder stored at 10 °C for 21 days, followed by 1 day at 25 °C. It was found that after storage Queen pineapple developed more IB than Smooth Cayenne pineapple. A negative correlation was found between the calcium content in both Queen ($r = -0.636$) and Smooth Cayenne ($r = -0.934$) pineapples. The effect of pre-harvest and postharvest calcium applications on IB of pineapple cv. Trad-see-thong was also studied. Fruit from plants sprayed with 0.1% calcium-boron solution combined with 150 kg/ha of calcium oxide dressing exhibited a 56% and 71% reduction of IB in the pulp and the core, respectively. In a postharvest study, calcium application by immersion of fruit stems in 1, 2 and 4% calcium chloride ($CaCl_2$) solutions at 25 °C (80-85% RH) for 18 hours reduced IB by 32%, 56% and 76% in pulp, respectively. However, the 2 and 4% $CaCl_2$ solutions caused a dark brown area in the fruit stem, which extended 2.5 cm into the core. Repeated experiments with 0-4% $CaCl_2$ solutions for 18 hours and 0.5-1.5% $CaCl_2$ solutions at 25 °C for 24-72 hours at 25 °C (80-85% RH) could not confirm the effect of postharvest calcium application on IB reduction. This suggests that calcium content is only one of the factors influencing IB in pineapples.

The relationship between the changes in endogenous gibberellin (GA) [gibberellin A_1 (GA_1), gibberellin A_3 (GA_3) and gibberellin A_4 (GA_4)] and abscisic acid (ABA) concentrations and IB in pineapple was also investigated in pineapple cvs. Trad-see-thong and Pattavia. The fruits were subjected to three different storage conditions for 21 days: 10 °C, 25 °C, or 10 °C followed by 1 day at 25 °C. In both cultivars, polyphenol oxidase (PPO) activity was found to correlate with IB and was highest in fruit transferred from 10 °C to 25 °C. Endogenous total GA ($GA_1 + GA_3 + GA_4$) concentrations were significantly increased after the fruit were transferred from 10 °C to 25 °C. In contrast, endogenous ABA concentrations were significantly increased during storage at 10 °C but decreased after the fruit were transferred from 10 °C to 25 °C. However, Trad-see-thong pineapple had higher endogenous total GA and ABA concentrations and developed more IB than those in Pattavia pineapple. In addition, both cultivars treated with 433 μM GA_3 for 5 min showed higher IB, PPO activity and ABA concentration than the control during storage at 25 °C. At 10 °C, GA_3 application significantly inhibited the increases in ABA concentration and PPO activity and the development of IB. The results suggest that GA and ABA are associated with IB but their roles are unclear.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to my advisor, Professor Dr. Jingtair Siriphanich for valuable advice and suggestions, reviewing the manuscript and his valuable encouragement throughout the course of my Ph.D. program. I also would to express my sincerest appreciation to Professor Dr. Satoru Kondo and Dr. Parson Saradhuldhat, my co-advisor, and also Dr. Kietsuda Luengwilai, the examination chairperson, and Dr. Lampan Khurnpoon, the external examiner, for their encouragement and assistance which were essential for completion of thesis.

I also would like to express my sincerest appreciation to Assistant Professor Dr. Katsuya Okawa, Associate Professor Dr. Hitoshi Ohara, Dr. Hataitip Nimitkeatkai, Dr. Monrudee Kittikorn and all members in the Pomology Laboratory of Graduate School of Horticulture, Chiba University, Japan, for their kindly suggestion and assistances for completion of thesis. I extent my thanks to Mrs. Chompoo Juntee for pineapple samples and research work in orchard and also to all graduate students in Postharvest Laboratory and staff of Postharvest Technology Center, Kasetsart University, Kamphaeng Sean Campus, Nakorn Pathom, Thailand for their hospitality and facilitating the instruments and materials during the research.

I am indebted to the Postharvest Technology Innovation Center, the KURDI, the JENESYS, Japan and the CASAF, NRU-KU, Thailand for awarding me a scholarship to undertake this study and financial support. I also extent my thanks to the Postharvest Technology Center, Kasetsart University Kamphaeng Sean Campus, Nakorn Pathom, Thailand for providing place and instruments for my experiments.

Finally, special appreciation is extended to my family, Pusittigul, whose encouragement and support contributed enormously throughout my study.

Issaya Pusittigul

July 2014

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LIST OF ABBREVIATIONS

1-MCP	=	1-methylcyclopropene
ABA	=	abscisic acid
ABA-d_6	=	3′,5′,5′,7′,7′,7′-hexadeuterated ABA
Ca^{2+}	=	calcium ion
$CaCl_2$	=	calcium chloride
CaO	=	calcium oxide
GA	=	gibberellins
GA_1	=	gibberellin A_1
GA_3	=	gibberellin A_3, gibberellic acid
GA_4	=	gibberellin A_4
GA_7	=	gibberellin A_7
GA13ox	=	GA 13-hydroxylase
GA20ox	=	GA 20-oxidase
GA3ox	=	GA 3-hydroxylase
GA2ox	=	GA 2-oxidase
GAMYB	=	GA-induced-MYB like transcription factor
GARC	=	elements of gibberellin response complex
GARE	=	gibberellin response element
CBF1	=	c-repeat binding factor
GC–MS-SIM	=	gas chromatography–mass spectrometry-selected ion monitoring
Ha	=	hectare
HPLC	=	high performance liquid chromatography
IB	=	Internal browning
LSD	=	least significant difference
LTRE	=	low-temperature-response-element
PPO	=	polyphenol oxidase
PVPP	=	polyvinylpolypyrolidone

EFFECTS OF CALCIUM, GIBBERELLINS AND ABSCISIC ACID ON INTERNAL BROWNING OF PINEAPPLE

INTRODUCTION

In Thailand, pineapple is one of the most important commercial fruit crops both as a fresh and processed product. In 2013, total pineapple exported were approximately 0.73 million metric tons, however fresh pineapple exported were approximately 1,646 metric tons which was less than 0.09% of total value of pineapple exported (Ministry of Commerce of Thailand, 2013). During the storage and transportation of fresh pineapple, the fruit must be kept at low temperature to delay senescence and minimize disease development. However, low temperature induces chilling injury resulting in internal browning (IB) of the pineapple, which develops first in the pulp near the core and later covers the whole pulp and core (Paull and Rohrbach, 1985). The IB incidence differs among cultivars. Kew (Smooth Cayenne group) pineapple is more resistance to chilling injury than Mauritius (Queen group) pineapple (Hewajulige *et al*., 2003). The commercial pineapple cultivars in Thailand include Pattavia (Smooth Cayenne group), a chilling tolerant cultivar, and Trad-see-thong (Queen group), a chilling susceptible cultivar (Nukuntornprakit and Siriphanich, 2005). Queen pineapple is only suitable for local consumption and for exportation by air because of its sensitivity to IB. Therefore, understanding the difference in the mechanism of IB development of both groups may lead to better ways in handling Queen pineapple or lead to a breeding program to improve its resistance to IB.

Calcium content is negatively correlated with IB symptoms in Queen and Smooth Cayenne pineapples (Hewajulige *et al*., 2003). Calcium maintains the stability of the membranes (Picchioni *et al*., 1995). Calcium oxide or calcium chloride application during fruit development reduces the IB development in Queen pineapples (Selvarajah *et al*., 1998; Herath *et al*., 2003). In Thailand, calcium content is negatively correlated with IB in Phulae (Queen group) and Phuket (Queen group) pineapples and Pattavia (Smooth Cayenne group) pineapple. However, calcium

content in Trad-see-thong (Queen group) and Sawee (Queen group) pineapples are not correlated with the IB all year round (Pitukwong *et al*., 2013). Calcium boron application during fruit development reduces the IB in Trad-see-thong (Queen group) pineapple (Sangudom *et al*., 2008). Calcium chloride application by peduncle infiltration reduces the IB development during the cool season (November) in Trad-see-thong (Queen group) pineapple while storing at 13 °C for 14 days (Youryon *et al*., 2013). However, there are no published works conducted on this aspect for other seasons. In addition, pre-harvest soil application and postharvest application by stem dipping on pineapple have not been reported.

Plant hormones, such as gibberellins (GA) and abscisic acid (ABA), have roles in various developmental and physiological processes in plants including fruit ripening and senescence (Kondo *et al*., 2002, 2003; Thomas and Hedden, 2006; Achard and Genschik, 2009). GA and ABA are also involved in responses to environmental stress such as low temperature. The decrease in the endogenous GA and the increase in endogenous ABA concentrations are associated with plant resistance to chilling stress in bamboo (*Neosinocalamus affinis*) (Zhang *et al*., 2012). ABA concentration, which is associated with the defense system in plants, increases chilling tolerant in zucchini squash (*Cucubita pepo* L.) (Wang, 1991). ABA, which plays a role in stress signal transduction, induces the secondary metabolite production against stress (Kondo *et al*., 2011).

Low temperature induces the activity of polyphenol oxidase (PPO), which is directly related to incidences of IB, in both Queen and Smooth Cayenne pineapples (Zhou *et al*., 2003a; Youryon *et al*., 2008). The increase in activity of PPO to chilling response correlates with the increases in *PINPPO1* and *PINPPO2* genes expression in Smooth Cayenne pineapple (Zhou *et al*., 2003a). The promoters of both *PPO* genes consist of complex sequence homologues to elements of gibberellin response complex (GARC). Gibberellic acid (GA_3) application increases IB, polyphenols (Zhou and Tan, 1992; You-Lin *et al*., 1997), PPO activity (Zhou and Tan, 1997) and induces *PPO* gene expression in Smooth Cayenne pineapple in the absence of chilling (Zhou *et al*., 2003b). Moreover, application of ABA, GA action inhibitor, decreases IB by delaying the increase in PPO activity in Smooth Cayenne pineapple after storage at chilling

temperature (Zhou and Pan, 1997). However, application of another GA biosynthesis inhibitor, paclobutrazol, fails to reduce the IB in Smooth Cayenne pineapple after storage at chilling temperature (O'Hare *et al*., 2001). Although GA inductions of *PINPPO1* and *PINPPO2* genes expression and PPO activity in pineapples have been reported, the changes of endogenous GA and ABA concentration during storage at low temperature have not been investigated. Nor has the GA effect on IB and PPO among different pineapple groups.

Therefore, determinations of the relation between calcium content and IB, the effects of pre- and post- harvest calcium applications on IB, the change of endogenous GA concentration and the effect of GA_3 on endogenous ABA concentration, PPO activity and IB of pineapples during storage at low temperature in Queen and Smooth Cayenne pineapples will increase the understanding of the mechanism of IB development in both pineapples during storage at low temperature.

OBJECTIVES

1. To determine the correlation between calcium content and IB of both Queen and Smooth Cayenne pineapples harvested from different planting locations in Thailand and the effects of pre- and postharvest calcium applications on IB development in Queen pineapples after being stored at low temperature.

2. To determine the correlation between changes in endogenous GA and ABA concentrations at low temperature storage and the changes in PPO activity and IB of both Queen and Smooth Cayenne pineapples after storage.

LITERATURE REVIEW

1. Pineapple and economic importance

Pineapple fruit (*Ananas comosus* L.), is a non-climacteric fruit, which belong to the family *Bromelliaceas* and is native to tropical America (Paull, 1997). Thailand is the world's largest pineapple producer followed by Costa Rica, Brazil, Philippines and Indonesia (Food and Agriculture Organization of United Nations, 2012). Pineapple, both as fresh and as processed products, is one of the most important commercial fruit commodities in Thailand. In 2013, total pineapple exports were approximately 0.73 million metric tons or 22,265 million baht in value. However fresh pineapple exports were approximately 1,646 metric tons or only 18.4 million baht in value, this was less than 0.09% of total value of pineapple exported. The export markets of Thailand's fresh pineapple include Singapore and China (Ministry of Commerce of Thailand, 2013).

Pineapple is a multiple fruit which develop from numerous sessile flowers to form the collective syncarpous fruit (Okimoto, 1948). Pineapple varieties are divided into 5 groups, including Abacaxi, Smooth Cayenne or Cayenne, Maipure or Perolera, Queen and Spanish, according to their morphological characteristic such as shape, weight, texture and taste of the fruit (Py *et al*., 1987; Leal and Soule, 1977). Pineapple fruit in the Queen group is small in size, with full yellow shell and golden-yellow pulp, sweet taste and crispy texture. Pineapple fruit in the Smooth Cayenne group is medium in size, juicy and pale-yellow pulp. Pineapple fruit in the Spanish group is small in size with golden-yellow pulp and poor taste (Py *et al*., 1987; Sripaoraya *et al*., 2001).

A study was conducted on pineapple cultivars in Thailand using the random amplified polymorphic DNA technique and it found that five pineapples cultivars, Phuket, Phulae, Trad-see-thong, Sawee and Petburi No.1, belonged to the Queen group, while two pineapples cultivars, Nanglae and Pattavia, belonged to the Smooth Cayenne group and two pineapples cultivars, Intrachidang and Intrachikow, belonged to the Spanish group. Phuket and Phulae pineapples, have high similarity coefficient, are

probably the same cultivars as shown in Figure 1 (modified from Sripaoraya *et al*., 2001; Popluechai *et al*., 2007).

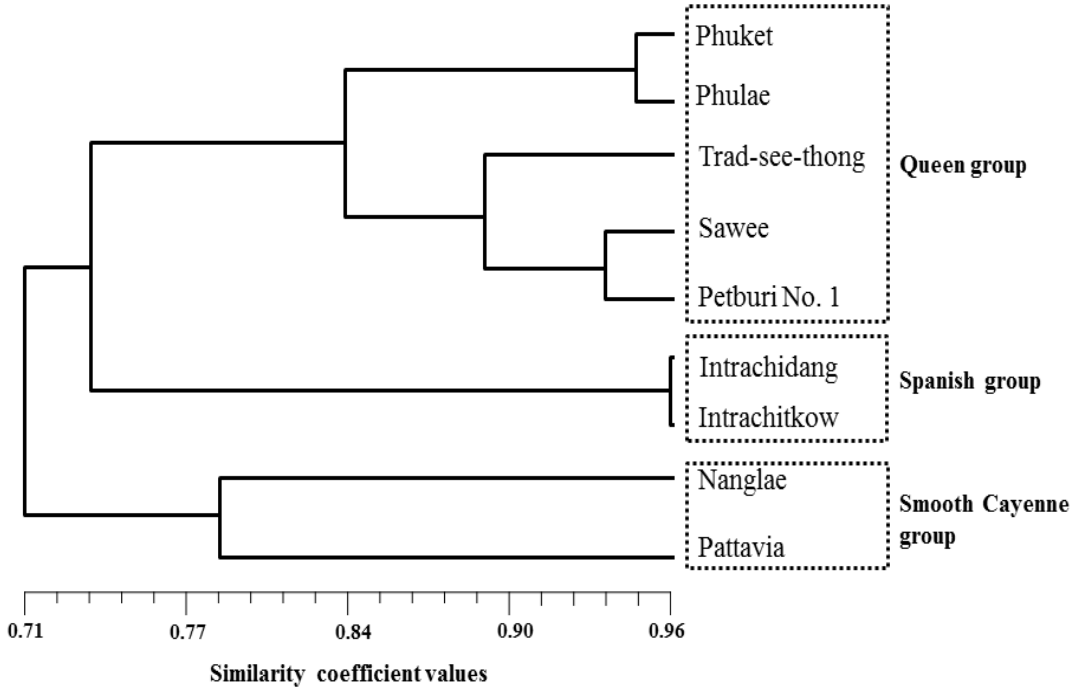

Figure 1 Dendrogram of genetic relationships between nine pineapples cultivars base on dice similarity coefficient values.

Source: Modified from Sripaoraya *et al*. (2001); Popluechai *et al*. (2007)

2. Chilling injury in pineapple

In general, the storage life of the pineapple is short. Fruit must be kept at low temperature to delay senescence and minimize disease development during storage and transportation. However, low temperature induces chilling injury resulting in (1) internal browning (IB), (2) browning and dulling of fruit, (3) failure of green-shelled fruit maturing to yellow-shelled fruit, (4) wilting, drying and discoloration of crown leaves, (5) water soaking of tissues and (6) increasing susceptibility to decay. The IB in pineapples occurs in the pulp near the core at first and then expands to the core and the whole pulp (Paull and Rohrbach, 1985).

Many hypotheses of chilling injury mechanism have been reported. The most interesting hypothesis of the chilling injury mechanism in pineapple is the free radical mechanism. Free radicals are induced by chilling stress and cause oxidative damage to membrane, DNA protein and lipid, the membrane is broken down and chilling injury occurs. However, antioxidants can inhibit or delay the oxidative damage by scavenging free radicals (Shewfelt and Del Rosario, 2000). The occurrence and development of IB increase in Smooth Cayenne pineapple fruit when fruit are transferred to 25 °C after storage at 12-13 °C for 17-21 days (Stewart *et al*., 2001; Zhou *et al*., 2003a).

The IB incidence and its development differ among cultivars. For example, Queen pineapple shows higher susceptibility to IB compared to Smooth Cayenne pineapple (Wilson Wijeratnam *et al*. 1993; Weerahewa and Adikaram, 2005a). The commercial pineapple cultivars in Thailand include Trad-see-thong and Phulae (Queen group), chilling-sensitive cultivars, and Patavia and Nanglae (Smooth Cayenne group), chilling-tolerant cultivars (Pimpimol and Siriphanich, 1993; Nukuntornprakit and Siriphanich, 2005; Siriphanich *et al*., 2005; Pitukwong *et al*., 2013; Setha *et al*., 2013; Youryon *et al*., 2013).

The IB incidence and its development also differ among fruit maturity stages. In Australia, Smooth Cayenne pineapple at mature stage (75% maturity as commercial fruit; specific gravity was approximately 0.920-0.960) shows higher susceptibility to IB than immature (specific gravity was less than 0.905) and over-mature (specific gravity is more than 1.000) stages (Zhou *et al*., 2003a). In Philippines, half-ripe fruit of Smooth Cayenne pineapple (35-70% yellow eyes) also has more IB than fruit harvested at color break stage (5-10% yellow eyes) (Soares *et al*., 2005). In addition in Thailand, half-ripe of Trad-see-thong (Queen) pineapple fruit (50 % yellow eyes) also has more IB and shorter storage life than mature green fruit (Buanong and Wongs-Aree, 2012).

Pre-harvest and postharvest procedures are reported to reduce IB in pineapples. Pre-harvest potassium or calcium applications also reduce IB development in both Queen and Smooth Cayenne pineapples (Selvarajah *et al*., 1998; Herath *et al*., 2003; Nanayakkara *et al*., 2005; Soares *et al*., 2005; Wilson Wijeratnam *et al*., 2006). For

postharvest procedures, heat treatment (32-43 °C) reduces the disorder (Teisson *et al*., 1979). A dip treatment in water at 38 °C for 60 minutes reduces IB in the pulp and core in Mauritius (Queen) pineapple (Weerahewa and Adikaram, 2005b). Modified atmosphere packaging and surface coating are the most common procedure and are used commercially. Waxing prevents Pattavia (Smooth Cayenne) pineapple from IB for shipment but for not longer than 3 weeks, this treatment is not effective enough to be used commercially for Trad-see-thong (Queen) pineapple (Paull and Rohrbach, 1985; Pimpimol and Siriphanich, 1993; Nimitkeatkai *et al*., 2006). However, sta-fresh 2952 waxing treatment reduces IB by reducing cell membrane permeability and malondialdehyde content, resulting in cell membrane damage, and so delays the decrease of ascorbic acid in Comte de Paris (Queen) pineapple (Hu *et al*., 2011). Additionally, postharvest calcium application also reduces IB in Trad-see-thong (Queen group) pineapple (Youryon *et al*., 2013).

In addition, postharvest 1-methylcyclopropene (1-MCP) application reduces IB symptoms in Queen pineapple (Selvarajah *et al*. 2001; Setha *et al*., 2013). Moreover, the applications of plant hormones reduce IB development, including gibberellic acid (GA_3) or abscisic acid (ABA) application in Smooth Cayenne pineapple (Zhou and Pan, 1997; O'Hare *et al*., 2001), methyl jasmonate application in Pattavia (Smooth Cayenne) pineapple (Nilprapruck *et al*., 2009) and salicylic acid application in Comte de Paris (Queen) pineapple (Lu *et al*., 2011).

3. Factors affecting internal browning

The IB can also be induced before harvest. Pineapple susceptibility to IB is not only influenced by the cultivar and maturity stage, but also climatic, weather and growing area conditions where they are grown and nutrients that pineapples obtained during their gowning period or after harvest (Abdullah, 2011).

3.1 Climatic, weather and growing area conditions

Climactic, harvest season, rainfall and growing area condition can also affect the susceptibility to IB of pineapple. Pineapples grown at different locations

with different climatic condition also have a different degree of IB (Abdullah, 2011). Pineapples grown in sub-tropical regions are prone to higher level of IB especially in the cool season (Leverington, 1971; Akamine *et al*., 1975; Teisson *et al*., 1979; Paull and Rohrbach, 1985; Kruger *et al*., 2000). 73-50 (hybrid) pineapple, which is harvested during the winter season in Hawaii, USA, shows more IB than fruit grown than those harvested in Queenland, Australia (Taniguchi *et al*., 2008).

Pineapple harvested in the autumn and winter seasons (form November to March), show more IB than fruit harvested in the summer season (August) in Smooth Cayenne and Yellow Mauritius (Queen) pineapples in China (You-Lin *et al*., 1997). In Australia, Smooth Cayenne pineapple also shows IB in the fruit maturing during the winter season. In addition, 73-50 (hybrid) pineapple harvested in the winter season (September) has a high incidence of IB but no IB development is found in fruit harvested in the spring season (December) (Youryon *et al*., 2012). Moreover, total rainfall also is related to IB incidence in Trad-see-thong pineapple but is not related to IB in Phulae, Phuket and Pattavia pineapples (Pitukwong *et al*., 2013).

Pineapple harvested from growing areas where it has been cloudy or partly shaded, showed more IB (Smith and Glennie, 1987). However, when the growing area is under rubber trees compared to growing in an open field, it has no greater influence on the IB development in Queen and Smooth Cayenne pineapples (Siriphanich, 2011).

3.2 Nutrients

3.2.1 Potassium

Potassium is one of essential macronutrients for plants which is involved in many important physiological and biochemical processes, including stomatal control and maintaining photosynthetic electron transport (Cakmak, 2005). Potassium is required for numerous plant growth processes, such as increasing crop yield and improving crop quality (Dull, 1971). Additionally, potassium also induces plant resistance to environmental stresses including light, salt, drought and low temperature stresses (Cakmak, 2005). Potassium increases antioxidant and antioxidant

enzyme activities. Potassium application increases malic acid in Mauritius (Queen) pineapple (Nanayakkara *et al*., 2005). Potassium application also increases the activities of ascorbic peroxidase and quaiacol peroxidase in bean plant (*Phaseolus vulgaris*) (Cakmak, 1994).

Potassium fertilizer applied during the fruit developmental stages reduces IB in pineapple (Nanayakkara *et al*., 2005; Soares *et al*., 2005). Potassium sulphate (K_2SO_4) application by foliar application at four week before harvest not only reduces IB symptoms but, also increases fruit potassium contents in the pulp and the core in Mauritius (Queen) pineapple (Nanayakkara *et al*., 2005). Potassium chloride (KCl) plus potassium oxide (K_2O) applications also increases fruit potassium content in leaves and reduces the activities of polyphenoloxidase and peroxidase (key enzymes in IB process) and IB symptoms in Smooth Cayenne pineapple (Soares *et al*., 2005).

3.2.2 Calcium

Calcium is considered to be an important mineral element that is involved in physiological process of plant cells, particularly in the maintenance of cell wall structure by stabilization of middle lamella as calcium pectate, a decrease in postharvest decay and incidence of physiological disorders and membrane function, it also regulates of cellular processes (Poovaiah and Reddy, 1987; Singh *et al*., 2007). Moreover, calcium is also involved in the regulation of various response of plant to environmental stresses (Braam *et al*, 1996). The increase in cytosolic calcium mediates plant responses to cold temperatures (Minorsky, 1985). Calcium maintains integrity and selective permeability and prevents solute leakage of membrane (Picchioni *et al*., 1995). A degradation product of lipid peroxidation, malondialdehyde content, increased in the calcium deficient in Panovy tomato plant (*Lycopersicon lycopersicum*) (Schmitz-Eiberger *et al*., 2002). The increase in calcium content reduces the activities of membrane degradation enzymes such as phospholipase D and lipoxygenase in cucumber fruit (*Cucumic sativus* L.) (Mao *et al*., 2007). Calcium also increases antioxidant and antioxidant enzyme activities. Calcium application increases ascorbic acid in Mauritius (Queen) pineapple (Herath *et al*., 2003), strawberry (*Fragaria* × *ananassa* Duch.) (Singh *et al*., 2007) and pomegranate (*Punica granatum* L.)

(Mirdehghan and Ghotbi, 2014). Calcium application also increases activities of glutathione reductase in quince plant (*Cydonia oblonga* Mill.), monodehydroascorbate reductase, dehydroascorbate reductase and superoxide dismutase in loquat plant (*Eribotria japonica* Lindl.) (Hernández *et al*., 2003).

Calcium is known as one of the factors influencing susceptibility to chilling injury in fruits and vegetables (Battey, 1990). High calcium content in plant tissue were observed to reduce the occurrence of chilling injury in avocado (*Persea Americana* Mill) (Chaplin and Scott, 1980) and Mauritius (Queen) and Kew (Smooth Cayenne) pineapples (Herath *et al*., 2003; Hewajulige *et al*., 2003). Calcium oxide (CaO) or calcium chloride ($CaCl_2$) application during fruit development reduces progression of IB in Mauritius (Queen) pineapple at low temperature (Selvarajah *et al*., 1998; Herath *et al*., 2003). $CaCl_2$ application during fruit development not only reduces IB symptoms but also increases endogenous calcium content in the pulp and the core in Mauritius (Queen) pineapple (Wilson Wijeratnam *et al*., 2006). Postharvest $CaCl_2$ application by peduncle infiltration also increases fruit calcium content and reduced the IB development in the cool season (November) Trad-see-thong (Queen group) pineapple during storage at low temperature (Youryon *et al*., 2013). Calcium content is negatively correlated with IB symptoms in both Mauritius (Queen) and Kew (Smooth Cayenne) pineapples (Hewajulige *et al*., 2003).

4. Plant growth regulator

Plant growth regulators or plant hormones, are a natural and/or synthetic organic material which in low concentration regulate plant growth and developmental process (Hooykaas *et al*., 1999; Roberts *et al*., 2002). The plant hormones are classified to auxins, abscisic acid (ABA), cytokinis, ethylene, gibberellins (GA) and jasmonates (Hooykaas *et al*., 1999).

4.1 Ethylene

Ethylene is gaseous plant hormone that affects the growth and development processes including ripening and senesces in many plants (Abeles *et al*.,

1992). Ethylene is synthesized from methionine in plant tissues as shown as shown in Figure 2 (Wang *et al*., 2002).

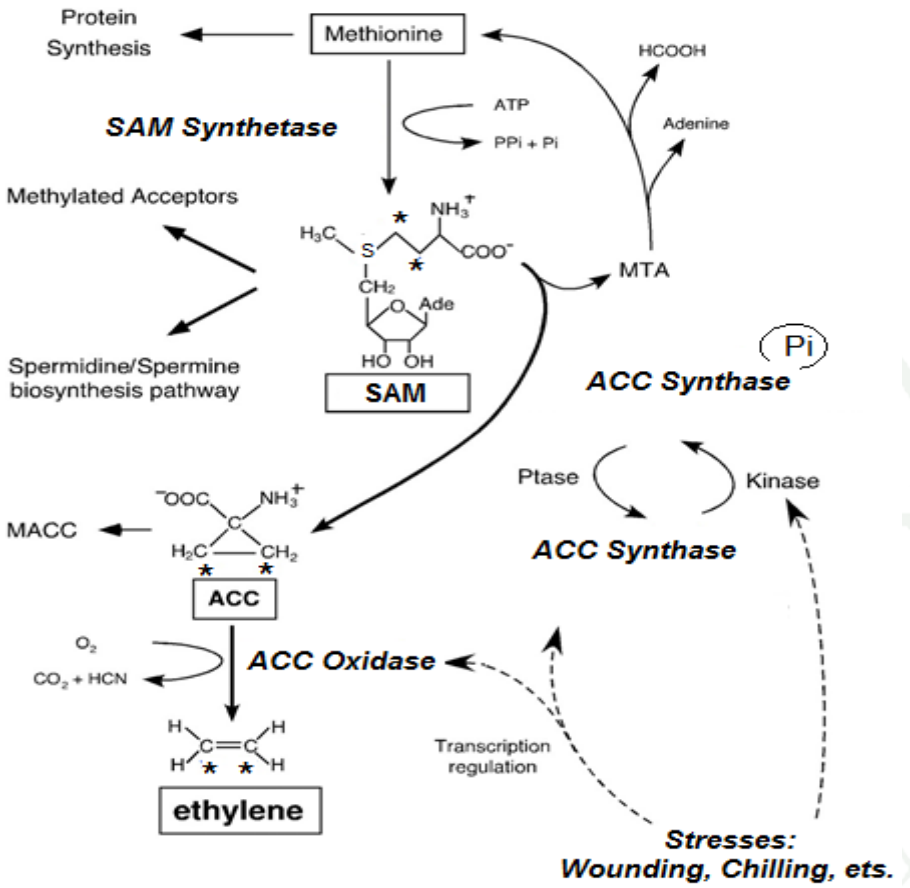

Figure 2 The ethylene biosynthesis pathway and regulation of ethylene. SAM: S-adenosyl-L-methionine; ACC: 1-aminocyclopropane-1-carboxylic acid; MACC: 1-malonylaminocyclo-propane-1-carboxylic acid; ATP: Adenosine triphosphate.

Source: Wang *et al.* (2006)

Endogenous ethylene production differs among of type tissues types, the plant species and the developmental stages of the plant, especially during ripening and senescence in climacteric fruit (Theologis, 1992). The expression of ethylene biosynthesis gene, *acacc-1* (ACC synthase), increases 16-fold in ripe pineapple compared to green fruit (Cazzonelli *et al*., 1998). Ethylene also acts as a stress

hormone, which is induced by many stress signals such as wounding, chemicals, drought, chilling temperature and pathogen Stress induced the ethylene production by increasing the expression of ACC synthase and ACC oxidase (Wang *et al*., 2002).

1-methylcyclopropene (1-MCP) is an inhibitor of ethylene action which interacts with ethylene receptors, competes with ethylene for binding sites and prevents ethylene-dependent responses (Sisler and Serek, 1997). 1-MCP inhibits the ripening and senescence of fruit and vegetables (Watkins, 2006). In South Africa, Selvarajah *et al*. (2001) reported that 1-MCP fumigation reduces the disorder in Queen Victoria (Queen) pineapple. In Thailand, 1-MCP application also reduces the increase in IB in Phulae (Queen) pineapple (Setha *et al*., 2013). However, postharvest treatment with 1-MCP has only a tendency to reduce IB in Queen and Smooth Cayenne pineapples but shows no significantl result (Siriphanich, 2011).

4.2 Gibberellins

GA are involved in many aspects of the higher plant developmental process, including seed germination and seed development, in flowers and fruits (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Olszewski *et al*., 2002; Thomas and Hedden, 2006; Achard and Genschik, 2009). More than a hundred GA of different chemical structures have been identified from higher plants (MacMillan, 2002), but only a few of them such as gibberellin A_1 (GA_1), gibberellin A_3 (GA_3), gibberellin A_4 (GA_4) and gibberellin A_7 (GA_7) are bioactive GA structures (Hedden and Phillips, 2000; Thomas and Hedden, 2006). GA are synthesized from the diterpene precursor, geranylgeranyl diphosphate (GGPP) in plastid as shown in Figure 3 (Thomas and Hedden, 2006).

Gibberellic acid (Gibberellin A_3, GA_3) application increases the fruit weight by enhancing flesh cell area in Comte de Paris (Queen) pineapple (Li *et al*., 2011). GA_3 also induced the expression of genes related to photosynthesis, chloroplast biogenesis, pathogen resistance and cold stress. GA_3 application also associated to fruit ripening by reducing the transcription of several ethylene-inducible genes, such as

carotenoid metabolic genes in Satsuma mandarin (*Citrus unshiu* Marc.) (Fujii *et al*., 2008).

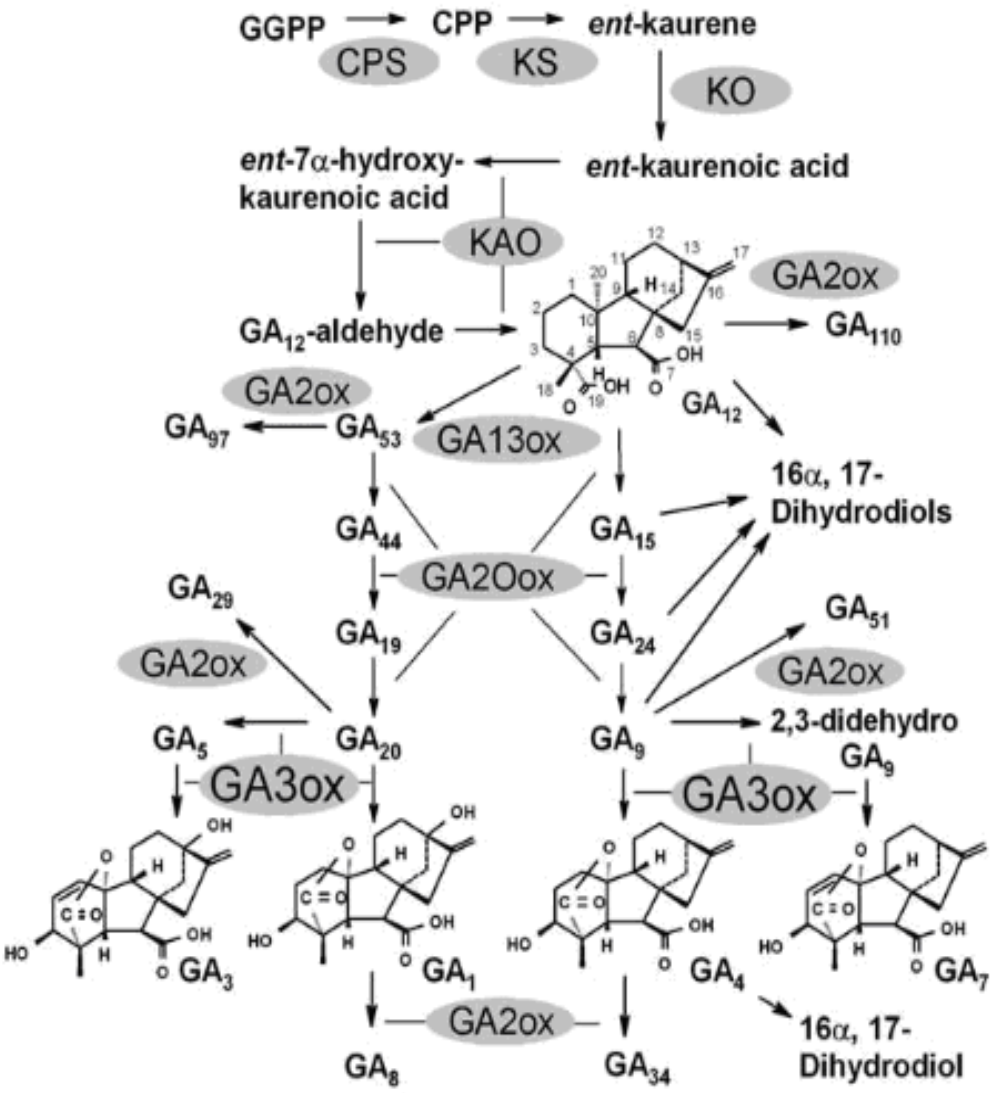

Figure 3 The GA biosynthesis and deactivation pathway in higher plants. GGPP: geranylgeranyl diphosphate; CPP: copalyl diphosphate. The enzymes catalysing highlighted in gray box are CPS: *ent*-copalyl diphosphate synthase; KS: *ent*-kaurene synthase; KO: *ent*-kaurene-oxidase; KAO *ent*-kaurenoic acid oxidase; GA13ox: GA 13-hydroxylase; GA20ox: GA 20-oxidase; GA3ox: GA 3-hydroxylase; GA2ox: GA 2-oxidase.

Source: Thomas and Hedden (2006)

Low temperature regulates GA metabolism, which differs in various species and developmental stages. Low temperature increases endogenous GA_3 concentration of wheat aleurone tissue (Singh and Paleg, 1985). Low temperature also increases endogenous GA_1 and GA_4 concentrations by stimulating the expression of *ent*-kaurene-oxidase (*AtKO1*), GA 20-oxidase (*AtGA20ox1*, *AtGA20ox2* and *AtGA20ox3*) and GA 3-hydroxylase (*AtGA3ox1*) genes that are GA biosynthesis genes and are required for increasing bioactive GA in *Arabidopsis thaliana* seed (Yamauchi *et al*., 2004). Although low temperature increases the relative level of GA biosynthesis, GA 20-oxidase (*GA20ox1*and *GA20ox3*) and GA 3-hydroxylase (*GA3ox2*), genes transcripts, also increases the relative levels of GA deactivation, GA 2-oxidase (*GA2ox1*, *GA2ox3* and *GA2ox6*), genes transcripts and decreases endogenous GA_1 and GA_4 concentrations in the rosette stage of *Arabidopsis thaliana* plant after 4 hours of cold treatment (Achard *et al*., 2008). Moreover, the decrease of endogenous GA_3 concentration in almond leaves (*Prunus* sp.) under low temperature (Yue and Wang, 2008) and the decrease of endogenous GA_1 and GA_4 concentrations in bamboo (*Neosinocalamus affinis*) under cold stress (Zhang *et al*., 2012) are also found. The chilling tolerance involves with the decrease of GA concentration in red kidney bean (*Phaseolus vulgaris*) (Reid *et al*., 1974) and the increase in ABA/GA ratio in alfalfa *(Medicago sativa* L.) seedlings (Waldman *et al*., 1975).

GA signaling pathway, GA first bind with receptor and promote binding DELLA protein (repressor of GA action) to GA-receptor-DELLA complex then enables degradation of GA-receptor-DELLA complex by proteasome and this then allows the occurrence of GA action (Achard and Genschik., 2009). DELLA protein inhibits the transcription of gene encoding to GA-induced-MYB like transcription factor (GAMYB) (Woodger *et al*., 2003). Moreover, the DELLA protein accumulates after cold treatment thereby improving freezing tolerance in *Arabidopsis thaliana* plant (Achard *et al*., 2008). GA application can also induces the increases in *GAMYB* gene transcription and protein levels within 2 hour and remains high over 12 hours after application in barley aleurone layers (*Hordeum vulgare* L.), (Gubler *et al*., 1995; Gubler *et al*., 2002). GAMYB is a transcriptional activator of GA regulated genes by binding with GARE of the α-amylase promoter in barley (Raventos *et al*., 1998).

In addition, ABA application is associated with the inhibition in expression of *HvGAMYB* gene of barley aleurone cells (*Hordeum vulgare*) (Gubler *et al*., 2002).

4.3 Abscisic acid

ABA is a plant hormone, which is involved in various physiological processes in plants including plant growth and development, seed dormancy and fruit ripening (Zeevaart and Creelman 1988; Kondo *et al.,* 2002). ABA is synthesized from carotenoids. In the early steps of ABA biosynthesis, caroteniods are synthesized from glyceraldehyde-3-phosphate (GPPP) and pyruvate in isopreniod pathway.
A β-carotene is converted to a zeaxanthin. The 9-*cis*-violaxanthin and 9'-*cis*-neoxanthin are changed to form xanthoxin by zeaxanthin epoxidase (ZEP), leaves the plastid and is subsequently converted to ABA in the cytosol as shown in Figure 4 (modified from Seo and Koshiba, 2002; RIKEN Plant Hormone Research Network, 2010).

Change in ABA, has been found to be associated with maturation and ripening of fruit, coincidental with changes in respiration and ethylene production. ABA is associated with fruit softening and sugar accumulation in the sweet cherry (*Prunus avium* L.) (Kondo and Gemma, 1993). The endogenous ABA concentration peaks immediately before fruit ripening of some fruit, including Granny smith apple (*Malus domestica*) (Lara and Vendrell, 2000) and Tsugaru apple (Kondo *et al*., 2001b), Seechompoo and Rongrien rambutans (*Nephelium lappaceum* L.) (Kondo *et al*., 2001a) and mangosteen (*Garcinia mangostana* L.) (Kondo *et al*., 2002). Furthermore, the endogenous ABA concentration increases before ripening and decreases until harvest in the non-climacteric sweet cherry (*Prunus avium* L.) (Kondo and Tomiyama, 1998).

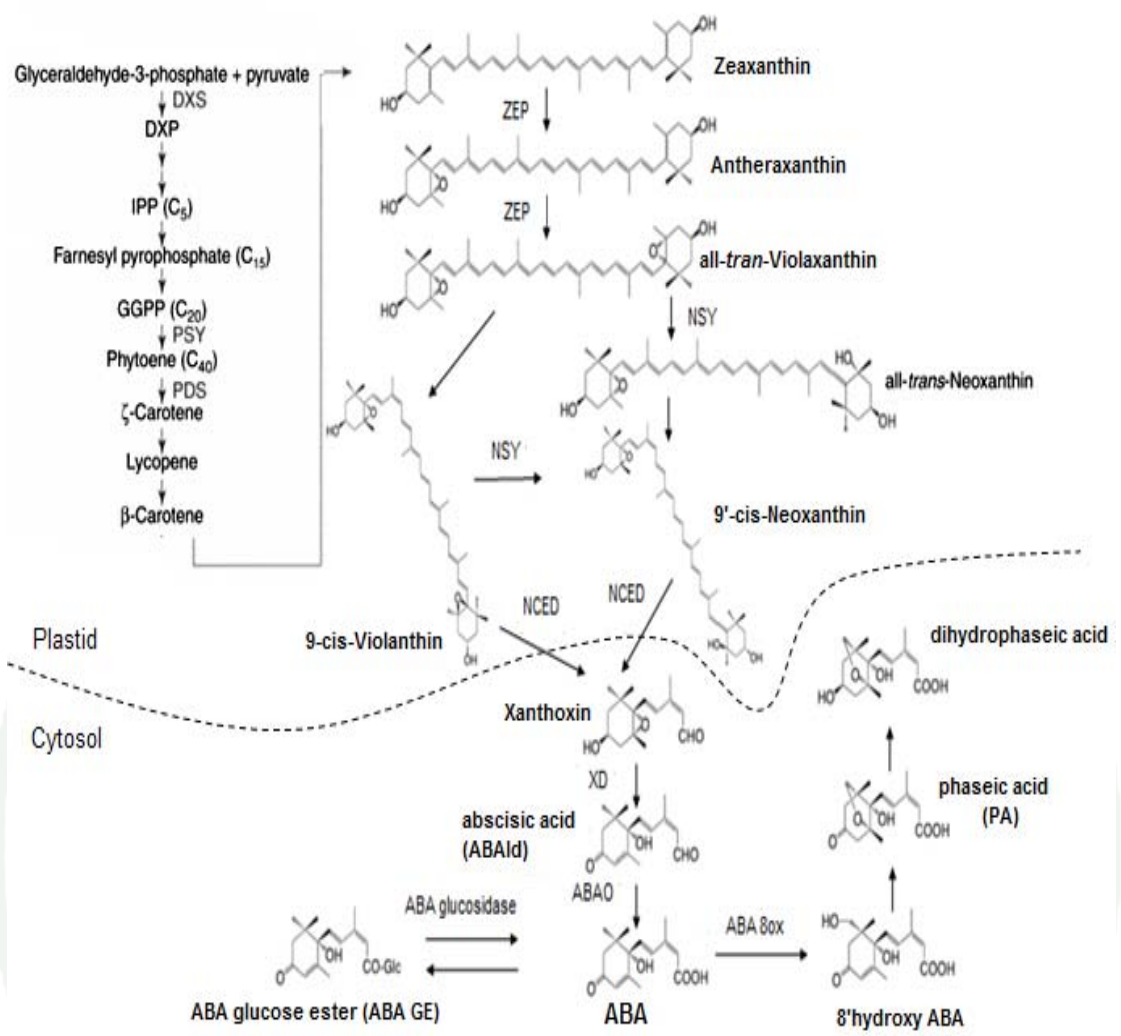

Figure 4 Abscisic acid biosynthesis and catabolism pathway in plant.
DXP: deoxyxylulose 5-phosphate; IPP: isopentenyl diphosphate; *GGPP:* geranylgeranyl diphosphate; *ABA:* abscisic acid. The enzymes catalysing these reactions are as followed: DXS: 1-deoxy-D-xylulose-5-phosphate; PSY: phytoene synthase; PDS: phytoene desturase; ZEP: zeaxanthin epoxidase; NSY: neoxanthin synthase; NCED: 9-cis-epoxycarotenoid dioxigenase; XD: xanthoxin dehydrogenase; ABAO: abscisic aldehyde oxidase; ABA 8ox: ABA 8´-hydorxylase.

Source: Modified from Seo and Koshiba (2002); RIKEN Plant Hormone Research Network (2010)

ABA plays a role against various environmental stresses such as low temperature. The increase in ABA concentration responses to cold stress of tomato leaves (*Lycopersicon esculentum*) (Kim *et al*. 2002). The increases in exogenous ABA concentrations are found under low temperature in zucchini squash (*Cucurbita pepo* L.) (Wang, 1991), almond leaves (*Prunus* sp.) (Yue and Wang, 2008), wheat seedling (*Triticum aestivum* L.) (Shakirova *et al*., 2009) and bamboo (*Neosinocalamus affinis*) (Zhang *et al*., 2012). Also in stored fruit, endogenous ABA concentration in the skin of mangosteen (*Garcinia mangostana* L.), which is associated with senescence and the degree of browning, increase at low temperature (Kondo *et al*., 2003).

5. Polyphenol oxidase

Polyphenol oxidase (PPO) is a copper containing enzyme which plays important roles in the browning of many fruits and vegetables (Mayer, 1986; Marshall *et al*., 2000; Mayer, 2006; He and Luo, 2007). PPO is located in chloroplast, which bound with thylakoid membranes and in non-green plastids (Vaughn and Duke, 1984; Vaughn *et al*., 1988) PPO is able to catalyze two reactions: hydroxylation of monophenols to *o*-diphenols (monophenol oxidase or cresolase activity) (EC 1. 14. 18. 1) and oxidation of *o*-diphenols to *o*-quinones (diphenol oxidase or catecholase activity) (EC. 1. 10. 3. 1). The hydroxylation and oxidation reactions use both oxygen molecules as a co-substrate (Mayer, 2006). Quinones, is a pigment precursors which is a strong electrophilic molecule, it reacts with the amino acid group and can polymerize, leading to the production of brown and black pigments, namely melanin, in many fruits and vegetables (Mayer, 1986; Marshall *et al*., 2000).

The substrates for the reaction include simple phenols such as catechol, gallic acid, chlorogenic acid and caffeic acid, and cinnamic acid derivatives such as dopamine and flavonids such as catechin and epicatechin (Fennema, 1975; He and Luo, 2007). Phenol is located in vacuole (Vaughn and duke, 1984). Reducing agent, which plays as anti-browning compounds such as ascorbic acid, citric acid and malic acid, reduces *o*-quinones to diphenol (colorless products), as shown in Figure 5 (Marshall *et al*., 2000).

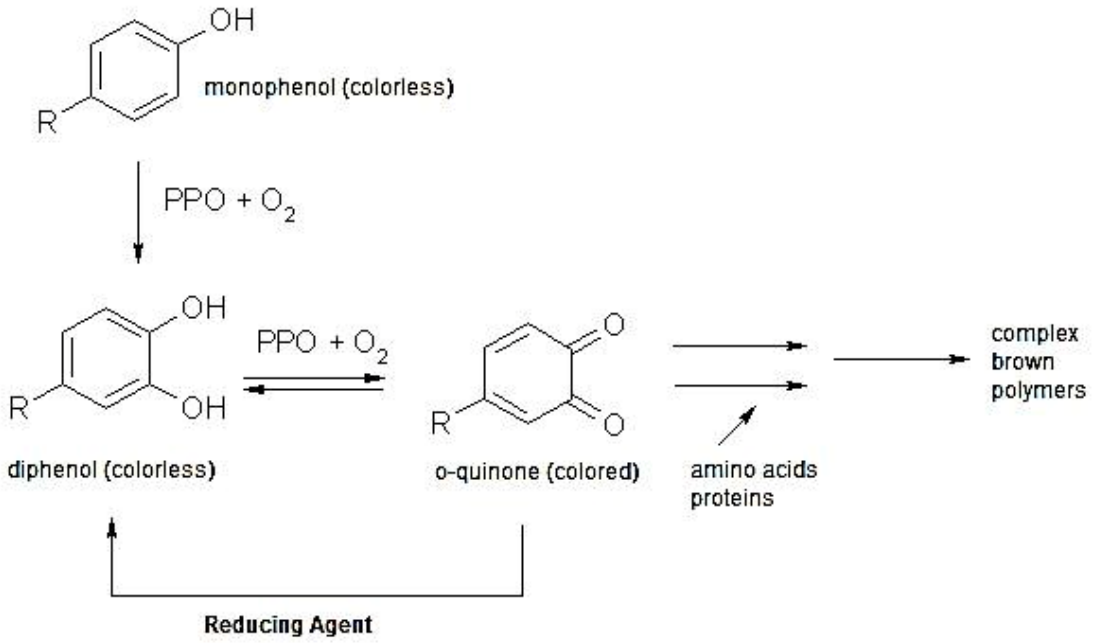

Figure 5 Enzymatic browning reaction induced by PPO and the role of reducing agent in the inhibition of enzymatic browning to reduce the quinones (pigment precursors) to colorless.

Source: Marshall *et al*. (2000)

The increase in PPO activities involves in fruit ripening and senescence and in response to chilling stress, where the membrane are damaged (Stewart *et al*., 2001; Zhou *et al*., 2003a; Mayer, 2006). PPO activity, is directly related to the IB development, dramatically increases following low temperature storage in both Queen and Smooth Cayenne pineapples (Zhou *et al*., 2003a; Youryon *et al*., 2008) and significantly increases after fruit transfer from low temperature to 25 °C in Queen pineapple (Zhou *et al*., 2003a). However, PPO is very low at harvest and remains low during storage at 25 °C in both Queen and Smooth Cayenne pineapples (Zhou *et al*., 2003a; Youryon *et al*., 2008). Low temperature induces PPO activity only in the pulp and the core, which have less PPO activity at harvest as compare with the skin and the crown leaves. The increase in PPO activity corresponded to chilling also is related to fruit maturity. PPO activity is higher in mature fruit compared to than immature and over mature fruit in Smooth Cayenne pineapple (Zhou *et al*., 2003a).

The expression of *PINPPO1* and *PINPPO2* genes increase during low temperature storage and after transfer from low temperature to 25 °C and this produces increase PPO activity and IB in Smooth Cayenne pineapple (Stewart *et al*., 2001). The expression level of the *PINPPO1* is higher than that in the *PINPPO2* gene during low temperature storage and after transfer from low temperature to 25 °C while low-temperature-response element (LTRE) (CCGAC) is only found in the *PINPPO2* promoter. However, the promoters of both, *PINPPO1* and *PINPPO2,* genes contain complex sequence homologues to elements of gibberellin response complex (GARC) (Zhou *et al*., 2003b). The GARC contains (1) the pyrimidine box (YCTTTTY, Y = C or T) which is the binding site of scutellum and aleurone-expressed DOF (SAD) and barley prolamin-box binding factor (BPBP) proteins, (2) the gibberellin response element (GARE) (TAACRRA, R = A or G) which is the binding site of barley gene encoding a novel DNA-binding protein (HRT) and gibberellin-responsive MYB transcription factor (GAMYB) proteins and (3) the box I element (TATCCAT) which is the binding site of single DNA-binding repeat MYB transcription factor (MYBS3) and multiubiquitin-chian-binding protein (MCB 1) proteins. Putative GAMYB binding sites (C/TAACC/AG/AA/CC/A) are also found in both, *PINPPO1* and *PINPPO2,* promoters (Jones *et al*., 1998; Zhou *et al*., 2003b; Rubio-Somoza *et al*., 2006; Woodger *et al*., 2007; Achard and Genschik., 2009). However, other environmental response elements such as light, ethylene, ABA, methyl jasmonate and auxin are not found in both, *PINPPO1* and *PINPPO2,* promoters in Smooth Cayenne pineapple (Zhou *et al*., 2003b).

GA_3 application increases IB, polyphenols (Zhou and Tan, 1992), catechol, chlorogenic acid, caffeic acid, PPO activity (Zhou and Tan, 1997) and induces PPO expression in Smooth Cayenne pineapple in the absence of chilling at 23 °C (Zhou *et al*., 2003b). Moreover, application of ABA, GA antagonist or inhibitor, decreases IB by delaying the increase in PPO activity in pineapple after storage at chilling temperature (Zhou and Pan, 1997).The hypothetically model of the GA signaling pathway involving induction of PPO synthesis is shown in Figure 6 (modified from Jones *et al*., 1998; Zhou *et al*., 2003b; Rubio-Somoza *et al*., 2006; Woodger *et al*., 2007; Achard and Genschik, 2009).

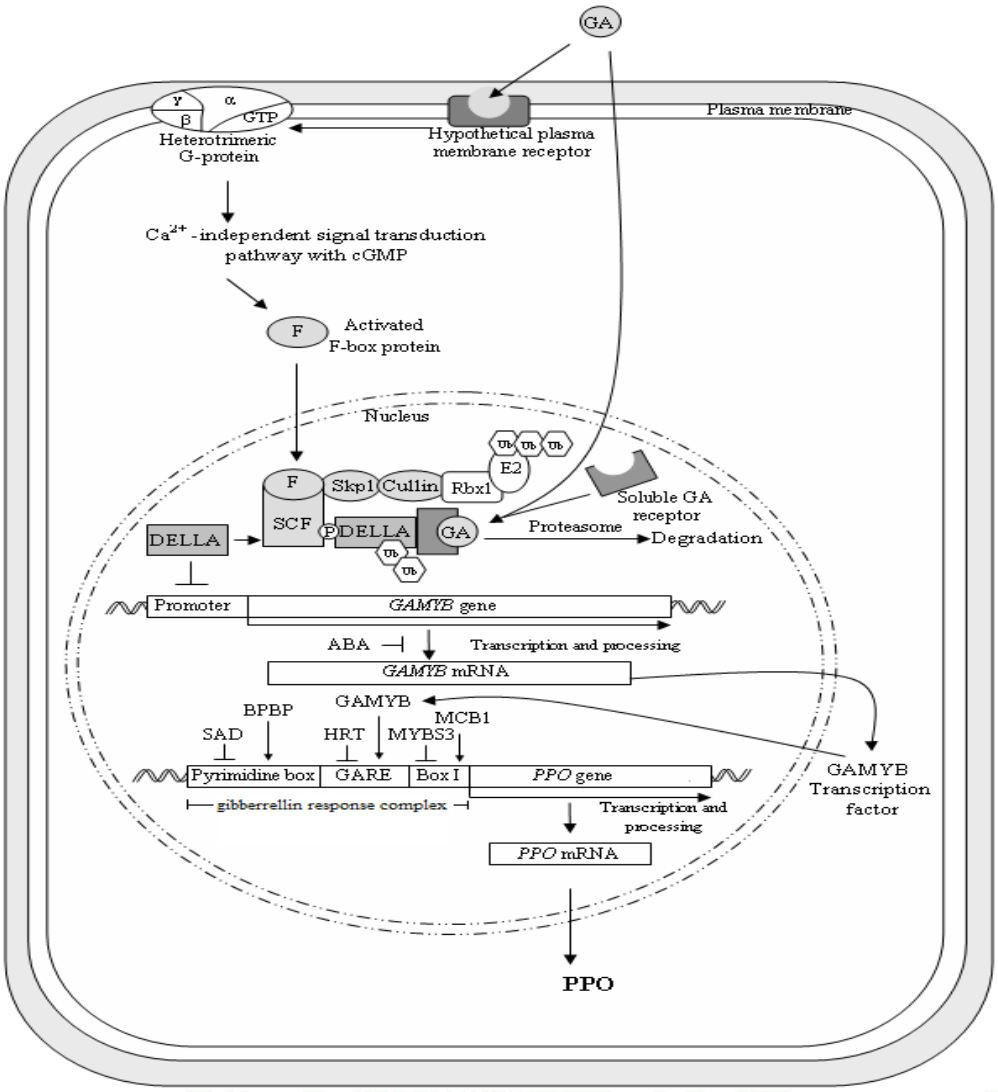

Figure 6 The hypothetically modified model of the GA signaling pathway which involved with the induction of PPO synthesis. GA: gibberrellins; ABA: abscisic acid; PPO: poluphenol oxidase. DELLA protein is a repressor of *GAMYB* gene. SAD: scutellum and aleurone-expressed DOF; BPBP: barlay prolamin-box binding factor; HRT: barley gene encoding a novel DNA-binding protein; GAMYB: gibberellin-responsive MYB transcription factor; MYBS3: single DNA-binding repeat MYB transcription factor; MCB 1: multiubiquitin-chian-binding protein.

Source: Modified from Jones *et al*. (1998); Zhou *et al*. (2003b); Rubio-Somoza *et al*. (2006); Woodger *et al*. (2007); Achard and Genschik (2009)

MATERIALS AND METHODS

1. Role of calcium on IB of pineapple

1.1 The relation between calcium content and IB of pineapples from different growing locations

1.1.1 2009 season

In April 2009, Pineapple (*Ananas comosus* L. Merr) at the mature green stage (140 and 150 days from forcing to harvest of Queen and Smooth Cayenne groups, respectively), having green peel and light-yellow pulp, the Queen and Smooth Cayenne pineapple were harvested from different growing locations in Thailand; pineapple cvs. Phulae (Queen) and Nanglae (Smooth Cayenne) from a plantation in Chiangrai Province, cvs. Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) from plantations in Rayong, Trad and Nakhon Pathom Provinces. Nineteen fruit of uniform size and maturity were gathered from each location. Before storage, nine fruits from each plantation were samples, an approximately 1 cm wide strip of tissue next to the core, as the pulp, and the core were taken from each fruit, this was then amalgamated into three samples (three fruit for one sample) and total calcium concentration of each sample was determined. Ten fruit were stored at 10 °C for 21 days of storage, followed by 1 day at 25 °C and evaluated for the percentage of internal browning area of the pulp and the core. One fruit was used as one replication. The experimental design was completely randomized design (CRD).

The data were combined and a linear regression analysis was performed, between the total calcium concentration and the percentage of internal browning area of Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) pineapples.

1.1.2 2010 season

In April 2010, Pineapple at mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, of pineapple cv. Trad-see-thong (Queen) were harvested from one plantation in Nakhon Ratchasima and Nakhon Pathom Provinces and two plantations in Phetchaburi Province, Thailand. Nineteen fruit of uniform size and maturity were used from each location. Before storage, the pulp and the core of nine fruit were sampled and then pooled into three samples (three fruit for one sample) for total calcium determination. The remaining ten fruit from each plantation were stored at 10 °C and evaluated for the percentage of internal browning area of the pulp and the core after 21 days of storage, followed by 1 day at 25 °C. One fruit was used as one replication. The experimental design was completely randomized design (CRD).

The data were combined and linear regression analysis between the total calcium concentration and the percentage of internal browning area was performed.

1.2 Effect of pre-harvest calcium applications on IB of Trad-see-thong pineapple

1.2.1 Effect of pre-harvest foliar calcium application on IB

Pineapple cv. Trad-see-thong (Queen) suckers were planted in double row pattern, with a spacing of 0.3 m (between plant along a row), 0.5 m (within double row) and 0.9 m (between double row), in Khao-sa-ming District, Trad Province, in November 2007 and harvested in December 2008. The plants were divided into 2 plots of 120 plants for 2 calcium treatments, as followed:

1) Control, drenching with 15-5-20 fertilizer, 10-15 g/plant at 3 and 6 months after planting.

2) Foliar application, drenching with fertilizer as in the control plus foliar sprayed with 0.1% calcium-boron solution, containing 6% calcium, 50 mL/plant, four applications before and after forcing at 15 days intervals, and another two applications after fruit set at 7 days intervals.

In all treatments, pineapple plants were forced 8 months after planting by foliar application with a solution containing 8 ml of 39.3% ai. Ethephon mixed with 300 g of urea in 200 L of water. Fruit of uniform size at the mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, were harvested. At harvest, the pulp and the core of nine fruit from each treatment were sampled and then pooled into three samples (three fruit for one sample) for total calcium determination. The remaining ten fruit from each treatment were stored at 10 °C for 21 days, followed by 1 day at 25 °C and then evaluated for the percentage of internal browning area of the pulp and the core, one fruit was used as one replication. The experimental design was completely randomized design (CRD)

1.2.2 Effect of pre-harvest soil calcium application on IB

Pineapple cv. Trad-see-thong suckers (Queen) were planted in double row pattern, with a spacing of 0.5 m (between plant along a row), 0.5 m (within double row) and 0.9 m (between double row), in Nakhon Ratchasima Province, in March 2009 and harvested in April 2010. The plants were divided into 3 plots of 180 plants for 3 calcium treatments, as followed:

1) Control, drenching with 15-5-20 fertilizer, 10-15 g/plant at 3 and 6 months after planting.

2) CaO 100, drenching as in the control together with top dressing CaO at 100 kg/ hectare (ha) , directly to the soil under the plant, two applications after 3 and 6 months from planting.

3) CaO 200, drenching as in the control together with top dressing CaO at 200 kg/ha, directly to the soil under the plant, two applications after 3 and 6 months from planting.

In all treatments, pineapple plants were forced 8 months after planting by foliar application with a solution containing 8 ml of 39.3% ai. Ethephon mixed with 300 g of urea in 200 L of water. Fruit of uniform size at the mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, were harvested. At harvest, the pulp and the core of nine fruit from each treatment were sampled and then pooled into three samples (three fruit for one sample) for total calcium determination. The remaining ten fruit from each treatment were stored at 10 °C for 21 days, followed by 1 day at 25 °C and evaluated for the percentage of internal browning area of the pulp and the core, one fruit was used as one replication. The experimental design was completely randomized design (CRD).

1.2.3 Effect of pre-harvest calcium application on IB

Pineapple cv. Trad-see-thong (Queen) suckers were planted in double row pattern, with a spacing of 0.3 m (between plant along a row), 0.5 m (within double row) and 0.9 m (between double row), in Khao-sa-ming District, Trad Province, in March 2008 and harvested in April 2009. The plants were divided into 20 plots of 1200 plants for 4 calcium treatments (5 plots per each calcium treatment), as followed:

1) Control, drenching with 15-5-20 fertilizer, 10-15 g/plant at 3 and 6 months after planting.

2) Foliar application, drenching with fertilizer as in the control plus foliar sprayed with 0.1% calcium-boron solution, containing 6% calcium, 50 mL/plant, four applications before and after forcing at 15 days intervals, and another two applications after fruit set at 7 days intervals.

3) Soil application, drenching as in the control together with top dressing CaO at 150 kg/ha, directly to the soil under the plant, two applications after 3 and 6 months from planting.

4) Foliar and soil application, combining calcium application as in the second and third treatment.

In all treatments, pineapple plants were forced 8 months after planting by foliar application with a solution containing 8 ml of 39.3% ai. Ethephon mixed with 300 g of urea in 200 L of water. Fruit of uniform size at the mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, were harvested. At harvest, the pulp and the core of twenty fruit from each treatment were sampled and then pooled into five samples (four fruit for one sample) for total calcium determination. Fifty fruit from each treatment were stored at 10 °C and evaluated for the percentage of internal browning area of the pulp and the core after 21 days of storage, followed by 1 day at 25 °C, one fruit was used as one replication. The experimental design was completely randomized design (CRD).

1.3 Effect of post-harvest calcium application on IB of Trad-see-thong Pineapple

1.3.1 Fixed submersion time in different calcium chloride solutions

Pineapple cv. Trad-see-thong (Queen), at the mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, was harvested in February 2009 from a plantation in Klang District, Rayong Province. Pineapples were transported to the laboratory within 6 hours. The fruit stems were trimmed to 5 cm in length and dipped (only the stem) in 0, 1, 2 and 4% $CaCl_2$ solutions at 25 °C for 18 hours (80±5% RH). Before and after immersion, nine fruit from each treatment were analyzed for total calcium content in the pulp and the core, as in experiment 1.1. Also, ten fruit from each treatment were stored at 10 °C for 21 days, followed by 1 day at 25 °C and then evaluated for the percentage of internal browning area of the pulp and the core, one fruit was used as one replication. The experiment was conducted in a completely randomized design (CRD). The experiment was repeated twice.

1.3.2 Fixed submersion time in calcium chloride solutions

Pineapple cv. Trad-see-thong (Queen), at the mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, was harvested in April 2009 from a plantation in Klang District, Rayong Province. Pineapples were transported to the laboratory within 6 hours. The fruit stems were trimmed to 7 cm in length and dipped (stem only) in 0 and 2 % $CaCl_2$ solutions at 25 °C for 18 hours (80±5% RH). Before and after immersion, nine fruit from each treatment were analyzed for total calcium content in the pulp and the core, as in experiment 1.1. Also, ten fruit from each treatment were stored at 10 °C for 21 days, followed by 1 day at 25 °C and then evaluated for the percentage of internal browning area of the pulp and the core, one fruit was used as one replication. The experiment was conducted in a completely randomized design (CRD). The experiments were repeated three times.

1.3.3 Different submersion duration in different calcium concentrations

Pineapple cv. Trad-see-thong (Queen), at the mature green stage (140 days from forcing to harvest), having green peel and light-yellow pulp, was harvested in June 2009 from a plantation in Klang District, Rayong Province. Pineapples were transported to the laboratory within 6 hours. The fruit stems were trimmed to 5 cm in length and dipped (stem only) in 0, 0.5, 1 and 1.5% $CaCl_2$ solutions at 25 °C for 24, 48 and 72 hours (80±5% RH). After application, ten fruit from each treatment were stored at 10 °C for 21 days, followed by 1 day at 25 °C and then evaluated for the percentage of internal browning area, one fruit was used as one replication. The experiment was conducted in 3 × 4 factorial in completely randomized design.

1.4 Evaluation procedures

1.4.1 Analysis of total calcium concentration

The extraction and analysis of calcium concentration was performed as reported by Kacar (1972) with some modifications. The pulp and the core of each fruit was sampled, dried and ground to fine powder. The 0.4 g dried sample [three replications for all experiment except for experiment 1.2.3, where five replications were used] was digested with 5 mL mixture of nitric acid: perchloric acid (2:1, v/v) at 70-270 °C. Then, the digested solution was adjusted with distill water to 50 mL. Calcium concentration was determined by using an atomic absorption spectrophotometer (model Vario 6; Analytic Jena AG, Jene, Germany).

1.4.2 Internal browning area

Fruit were cut longitudinally into two halves and the IB area of the pulp and the core were visually estimated on the percentage of 0 to 100%, where 0% = none, 1-25% = slight IB, 26-50% = moderate IB, 51-75% = moderately severe IB, 76-100% = severe IB.

2. The role of endogenous GA and ABA in IB development in pineapple

2.1 The changes of endogenous GA and ABA concentrations and IB of pineapple

Pineapple cvs. Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) were harvested at the mature green stage (140 and 150 days from forcing to harvest of Trad-see-thong (Queen) and Pattavia (Smooth Cayenne), respectively), from a plantation in Nakhon Ratchasima Province and transported to the laboratory within 6 hours. Three groups were created.

1) 10 ºC, the fruit were stored at 10 °C (85±5% RH) for 21 days

2) 10 ºC + 1 d 25 °C, the fruit were stored at 10 °C (85±5% RH) but were subjected to 25 °C for one day on either the 7^{th}, 14^{th}, 21^{st} day.

3) 25 °C, the fruit were stored at 25 °C (85±5% RH) for 21 days.

Ten fruit were randomly sampled and immediately sliced longitudinally to evaluate for IB. Approximately 1 cm wide strip of pulp next to the core was sampled, immediately frozen in liquid N_2 and kept at -80 °C for PPO activity determination. The frozen pulp was also freeze-dried and kept at -80 °C for endogenous ABA, GA_1, GA_3 and GA_4 concentrations analysis at Chiba University, Chiba, Japan. The experimental design was completely randomized design (CRD).

2.2 Effect of GA_3 application on IB during storage at 25 °C

Pineapple cvs. Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) at the mature green stage (140 and 150 days from forcing to harvest of Trad-see-thong (Queen) and Pattavia (Smooth Cayenne), respectively), from a plantation in Nakhon Ratchasima Province were dipped in 433 μM (150 mg/L) GA_3 for 5 minutes and stored at 25 °C (85±5% RH) for 21 days. Untreated control fruit were dipped in distilled water for 5 min. Ten fruit were randomly sampled at 7 days intervals to determine IB, PPO activity and endogenous ABA concentration, as in experiment 2.1. The experimental design was completely randomized design (CRD).

2.3 Effect of GA_3 application on IB during storage at 10 °C

Pineapple cvs. Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) at the mature green stage (140 and 150 days from forcing to harvest of Trad-see-thong (Queen) and Pattavia (Smooth Cayenne), respectively, from a plantation in Nakhon Ratchasima Province were dipped in 433 μM (150 mg/L) GA_3 for 5 minutes and stored at 10 °C (85±5% RH) for 21 days. Untreated control fruit were dipped in distilled water for 5 min. Ten fruit were randomly sampled at 7 days intervals to

determine IB, PPO activity and endogenous ABA concentration, as experiment 2.1. The experimental design was completely randomized design (CRD).

2.4 Evaluation procedures

2.4.1 Internal browning area

Fruit were cut longitudinally into two halves and the IB area was visually estimated on the percentage of 0 to 100%, where 0% = none, 1-25% = slight IB, 26-50% = moderate IB, 51-75% = moderately severe IB, 76-100% = severe IB.

2.4.2 Analysis of PPO activity

PPO was extracted and assayed according to the Benjamin and Montgomery method (1973) with some modifications. The fresh samples [three 2.5 g samples taken from ten fruit] were homogenized in 5 mL 0.1 M phosphate buffer, pH 7.3. The homogenate was centrifuged at 12,000×*g* for 10 min at 4 °C. The supernatant was used to measure the activity of the enzyme. The reaction mixture contained 1.2 mL of 0.1 M sodium phosphate buffer (pH 7.0), 0.7 mL of 0.1 M catechol and 0.1 mL of enzyme extract. The increase in absorbance at 420 nm was recorded for 3 minutes using a spectrophotometer (Model GENESYS 10 UV; Thermo Fisher Science Inc., Wisconsin, USA). One unit of enzyme activity was defined as the amount of enzyme that caused a change of absorbance per minute.

Protein content was determined according to the Bradford method (1976) at 595 nm with a spectrophotometer (Model GENESYS 10 UV; Thermo Fisher Science Inc., Wisconsin, USA), using bovine serum albumin as a standard. Specific activity of the PPO was expressed as units per mg protein.

2.4.3 Quantitative analysis of endogenous GAs

Endogenous gibberellins were identified and quantified according to the method of Goto *et al*. (1989) with some modifications. The freeze-dried samples [three 2 g samples taken from ten fruit] were homogenized in 20 mL 80% (v/v) methanol with 200 ng each of [$^2H_2$] GA_1, [$^2H_2$] GA_3 and [$^2H_2$] GA_4 as an internal standard. The homogenate was filtered and the residue was re-extracted twice with 20 mL 80% (v/v) methanol. The methanol extract was combined and evaporated to reduce to the aqueous phase. The supernatant was altered to pH 2.5 with 6 M hydrochloric acid and partitioned three-times with 20 mL ethyl acetate. The combined ethyl acetate extract was partitioned twice with 30 mL of 0.5 M potassium phosphate buffer, pH 8.3. The combined aqueous extract was altered to pH 2.5 with 6 M hydrochloric acid and partitioned three-times with 30 mL ethyl acetate. The combined ethyl acetate extract was passed through anhydrous sodium sulfate (9 g) for dehydration, evaporated to dryness, re-dissolved three-times in 1 mL ethyl acetate and dried *in vacuo*. The residue was re-dissolved in 6 mL of 0.1 M dipotassium hydrogenphosphate (pH 8.0) and loaded into a polyvinylpolypyrolidone (PVPP) column. The column was eluted with 0.1 M dipotassium hydrogenphosphate (pH 8.0). The collected eluate was adjusted to pH 2.5 with 6 M hydrochloric acid and partitioned three-times with 30 mL ethyl acetate. The combined ethyl acetate extract was passed through anhydrous sodium sulfate (9 g) for dehydration, evaporated to dryness, re-dissolved three-times in 1 mL ethyl acetate and dried *in vacuo*. The PVPP residue was dissolved in 3 mL of 50% (v/v) methanol and passed through individual Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, USA). Each cartridge was eluted three-times with 2 mL of 80% (v/v) methanol. The combined eluate was evaporated to dryness. The Sep-Pak C_{18} residue was dissolved in 80% (v/v) methanol, filtered and fractionated by high performance liquid chromatography (HPLC) (model L-7300; Hitachi High-Technologies Corp., Tokyo, Japan) using a Senshu-Pak ODS 4253D column (10 mm i.d. × 250 mm) and detected by UV absorption at 210 nm. The GA_1, GA_3 and GA_4 were collected from 9 - 28 min evaporated to dryness, redissolved three-times in 0.5 mL methanol and dried *in vacuo*. The residue was

redissolved with 1 mL of 10% (v/v) methanol in diethyl ether, methylated with diazomethane, trimethylsilylated with Deriva-sil (20 µL; Chrompack Inc., Raritan, NJ, USA) at 70 °C for 10 min, dried *in vacuo* and identified by gas chromatography–mass spectrometry-selected ion monitoring (GC–MS-SIM; model QP5000; Shimadzu, Kyoto, Japan) using a InertCap 1MS column (GL Sciences, Tokyo, Japan; 0.25 mm i.d. × 30 m, 0.25 µm film thickness) and linear Helium flow at 50.2 cm s^{-1}. The column temperature was a step gradient of 60 ºC for 2 min, then 60 to 270 ºC at 10 ºC min^{-1}, and 270 ºC for 35 min. The endogenous concentration of GA_1, GA_3 and GA_4 were calculated from the peak areas ratios of non-deuterated to deuterated GAs at as *m/z* 330/332, 237/239 and 284/286, respectively.

2.4.4 Analysis of endogenous ABA concentration

The extraction and quantification of endogenous ABA was performed as reported by Setha and Kondo (2009) with some modifications. The freeze-dried samples (three 1 g samples taken from ten fruits) were homogenized in 20 mL of cold 80% (v/v) methanol with 200 ng 3′,5′,5′,7′,7′,7′-hexadeuterated ABA (ABA-d_6) as an internal standard. The homogenate was centrifuged, filtered through filter paper No. 5 (pore size 4 nm, diameter 60 mm; Kiriyama Glass Works Co., Tokyo, Japan), and evaporated to reduce to the aqueous phase. The aqueous residue was adjusted to pH 2.5 with 0.1 M hydrochloric acid and partitioned three-times with 20 mL ethyl acetate. The ethyl acetate extract was combined, evaporated to dryness, re-dissolved three-times in 1 mL ethyl acetate. The solvent was dried and the residue was re-dissolved in 1 mL of 4.8 M acetonitrile containing 20 mM acetic acid. The solution was filtered through nitrocellulose filter (pore size 0.22 µm; EMD Millipore Corp., MA, USA) and fractionated by HPLC (model PU-980 Gulliver series; Japan Spectroscopic, Tokyo, Japan); using an ODS Mightysil RP-18 column (Kanto Chemical, Tokyo, Japan; 4.6 mm i.d. × 250 mm) with a gradient of 4.8 to 9.6 M acetonitrile containing 20 mM acetic acid over a 30 minutes period at a flow rate of 1.3 mL $minutes^{-1}$, and detected by UV absorption at 254 nm. The fraction containing ABA and internal standard was collected, evaporated to dryness, re-dissolved three-times in 0.5 mL methanol and dried *in vacuo*. The residue was re-dissolved with 1 mL

of 10% (v/v) methanol in diethyl ether, and then methylated with diazomethane for 10 minutes. The methyl ester of ABA was quantified and identified by using GC–MS-SIM (model QP5000; Shimadzu, Kyoto, Japan) using a InertCap 1MS column (GL Sciences, Tokyo, Japan; 0.25 mm i.d. × 30 m, 0.25 μm film thickness) and linear Helium flow at 50.2 cm s^{-1}. The column temperature was a step gradient of 60 ºC for 2 min, then 60 to 270 ºC at 10 ºC $minuate^{-1}$, and 270 ºC for 35 minutes. The ions were measured as ABA-d_0 methyl ester/ ABA-d_6 methyl ester at m/z 190, 260, 194 and 264. The endogenous ABA concentration was calculated from the peak area ratio of m/z 190 (ABA-d_0) /194 (ABA-d_6). Fragmentation ion patterns were compared with those of the chemical standard in total ion monitoring mode to identify ABA methyl ester in the samples.

3. Statistical analysis

All data was subjected to analysis of variance procedures by ANOVA, significance of the differences between means were estimated by Duncan's new multiple range test (DMRT) at $P \leq 0.05$, in experiment 1 and separated by Fisher's least significant difference (LSD) at $P \leq 0.05$, in experiment 2, using SPSS program version 16. Data are presented as means ± standard error (SE). Pearson correlation coefficients (r) between calcium content and IB were also estimated, in experiment 1.1.

RESULTS AND DISCUSSION

Results

1. Role of calcium on IB of pineapple

1.1 The correlation between calcium content and IB of pineapples from different growing locations

1.1.1 2009 season

In both Queen and Smooth Cayenne pineapples groups harvested from different growing locations, total calcium contents in the pulp were higher than those in the core, although were not all significantly different. Total calcium contents in the pulp of Trad-see-thong (Queen) pineapple harvested from Nakhon Pathom and Pattavia (Smooth Cayenne) pineapples harvested from Trad and Nakhon Pathom were significantly higher than those in the core (Figure 7A and 7B). After storage at 10 °C for 21 days followed by 1 day at 25 °C, the Queen pineapple group had percentages of IB area significantly higher in the core (78-80% IB of the core area) than those in the pulp (26-50% IB of the pulp area) in fruit harvested from Rayong and Trad. However, in the Smooth cayenne pineapple group, the percentages of IB area were significantly higher in the pulp (33% IB of the pulp area) than those in the core (11% IB of the core area) in fruit harvested from Rayong. The percentages of IB area were significantly higher in the Queen pineapple than those in the Smooth Cayenne pineapple harvested from Rayong and Trad, but were not significantly different in fruit harvested from Chiangrai and Nakhon Pathom (Figure 7C and 7D).

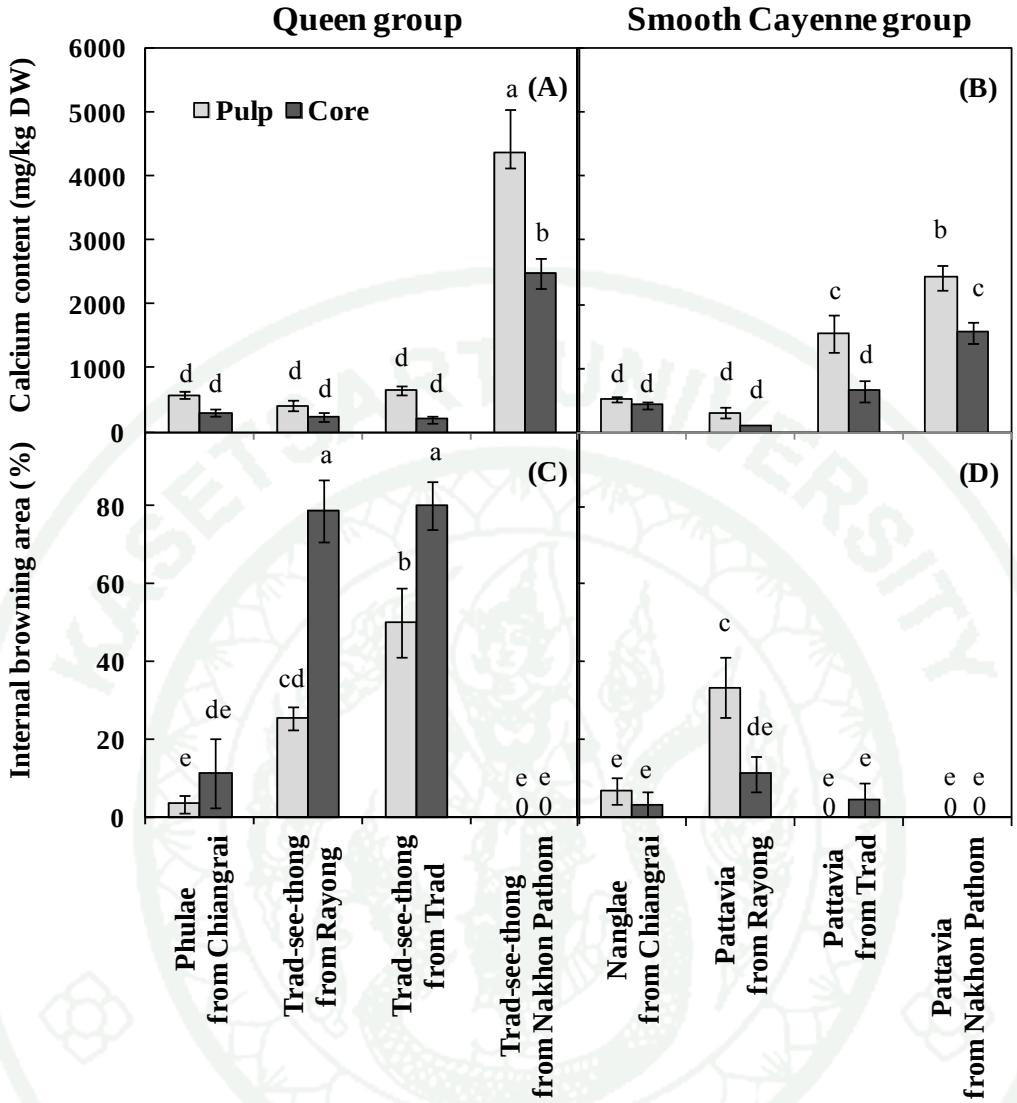

Figure 7 Total calcium contents before storage (A and B) and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C (C and D) in the pulp and the core of Queen (A and C) and Smooth Cayenne (B and D) pineapples harvested from four different locations. Data are means ± SE of three replications (A and B) and ten replications (C and D). Bar with the different letters in the same row are significantly different at $P \leq 0.05$.

Within the same group of pineapple, total calcium contents in the pulp were negatively related with the IB. Queen pineapple harvested from Nakhon Pathom had more than 4,300 and 2,400 mg/kg DW of calcium in the pulp and the core, respectively, and no IB was found after low temperature storage. Queen pineapples harvested from Chiangrai, Rayong and Trad, had less than 700 and 200 mg/kg DW of calcium in the pulp and the core, respectively, and showed moderate to severe IB symptoms (26-50% IB of the pulp area and 79-80% IB of the core area) except those harvested from Chiangrai which developed only slight IB symptom (3% IB of the pulp area and 11% IB of the core area).

Smooth Cayenne pineapples harvested from Nakhon Pathom and Trad had more than 1,500 and 650 mg/kg DW of calcium in the pulp and the core, respectively, and developed very little or no IB symptom (0% IB of the pulp area and 0-4% IB of the core area). Smooth Cayenne pineapples harvested from Chiangrai had less than 600 and 400 mg/kg DW of calcium in the pulp and the core, respectively, and developed slight IB symptoms (7% IB of the pulp area and 3% IB of the core area).

There were significant negative correlations between total calcium content and IB of Queen and Smooth Cayenne pineapples (Figure 8). There was a moderate negative correlation between total calcium content and IB of Queen pineapple ($r = -0.636$, $P \leq 0.05$), as showed in Figure 8A (Notice that the data area is poorly distributed), and there was a highly negative correlation between total calcium content and IB of Smooth Cayenne pineapple ($r = -0.934$, $P \leq 0.01$), as shown in Figure 8B.

Also within the same cultivar, there were significant negative correlations between total calcium content and IB of Trad-see-thong and Pattavia pineapples (Figure 9). There were strong negative correlations between total calcium content and IB of Trad-see-thong ($r = -0.966$, $P \leq 0.01$), as shown in Figure 9A (Notice that the data area is poorly distributed), and Pattavia ($r = -0.935$, $P \leq 0.01$), as showed in Figure 9B.

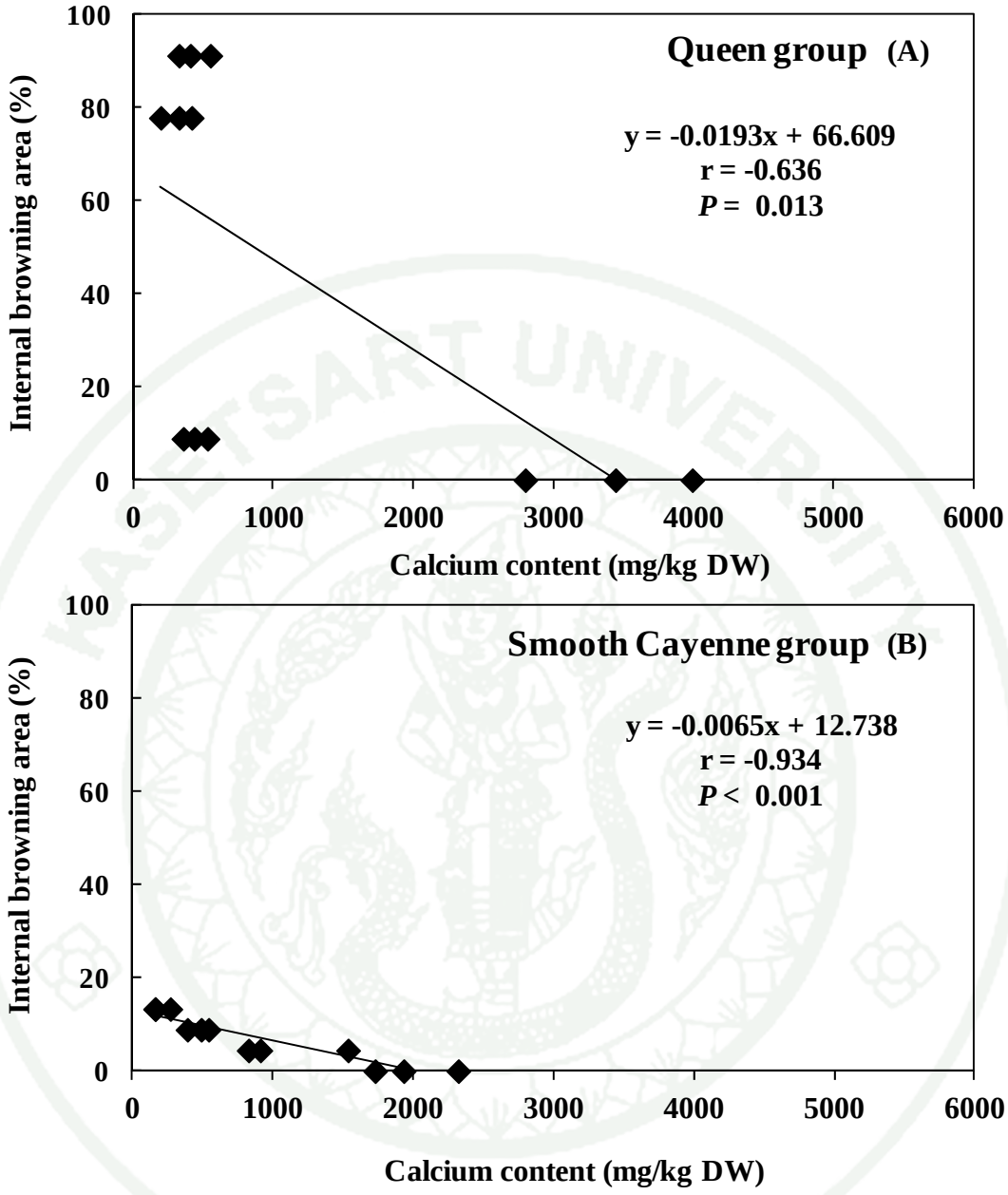

Figure 8 Correlations between total calcium content before storage and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Queen (A) and Smooth Cayenne (B) pineapples.

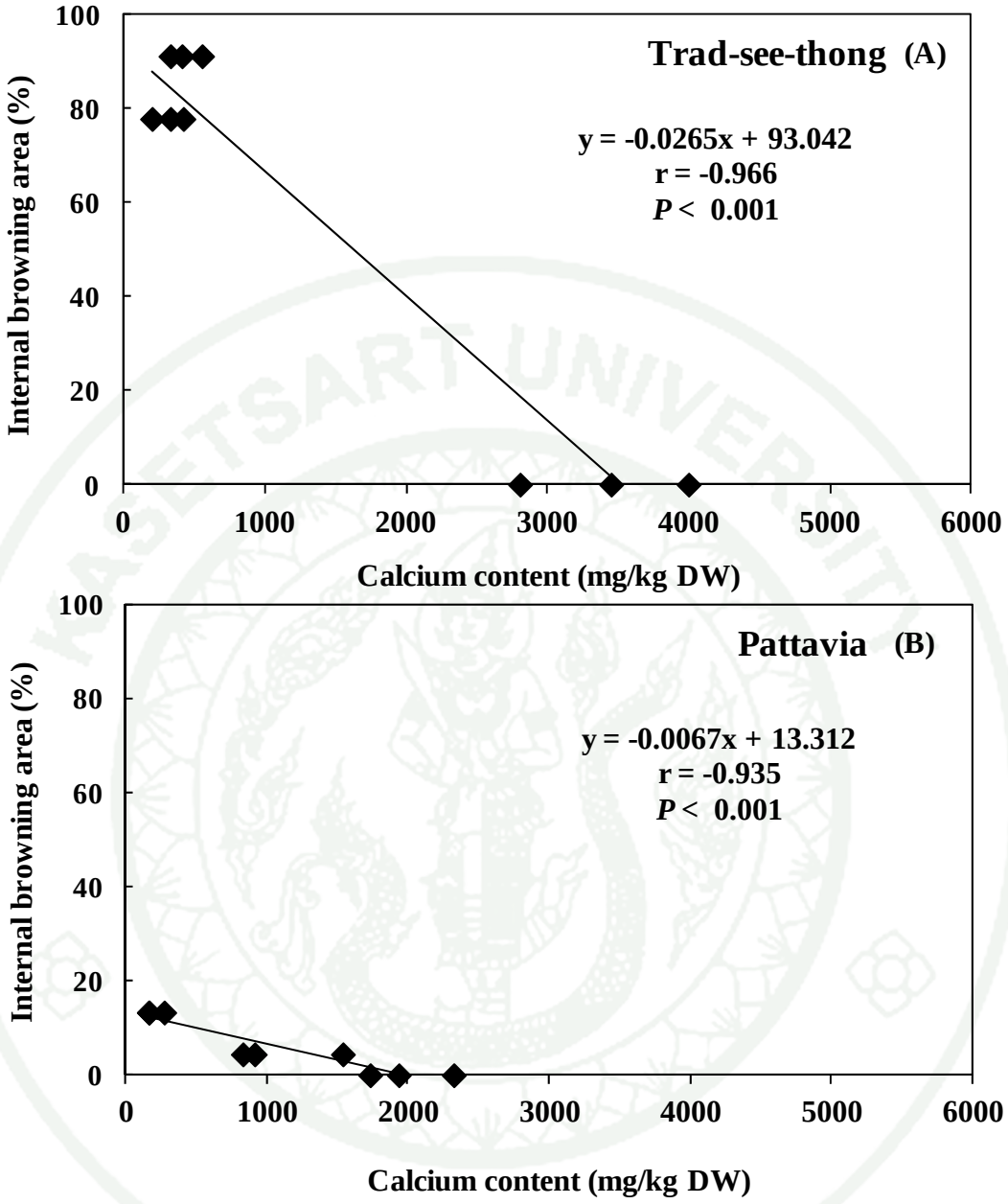

Figure 9 Correlations between total calcium content before storage and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong (A) and Pattavia (B) pineapples.

1.1.2 2011 season

In Trad-see-thong pineapples, the total calcium contents in the pulp were higher than those in the core, especially in the pulp of fruit harvested from Nakhon Pathom was significantly higher than that in the core (Figure 10A). Total calcium contents were related to IB development (Figure 10B). After storage at 10 °C for 21 days followed by a day at 25 °C, the percentage of IB was significantly the highest in fruit harvested from plantation 1 in Phetchaburi (50% IB of the pulp area and 58% IB of the core area) and the lowest in fruit from Nakhon Pathom (18% IB of the pulp and 28 % IB of the core area). Trad-see-thong pineapple from Nakhon Pathom had more than 2,300 and 1,100 mg/kg DW of calcium in the pulp and core, respectively, and developed slight to moderate IB after low temperature storage. Trad-see-thong pineapples harvested from plantation 1 and 2 in Phetchaburi and Nakhon Ratchasima had less than 400 and 290 mg/kg DW of calcium in the pulp and the core, respectively, and developed slight to moderately severe IB symptom (24-50 % IB of the pulp area and 36-58% IB of the core area).

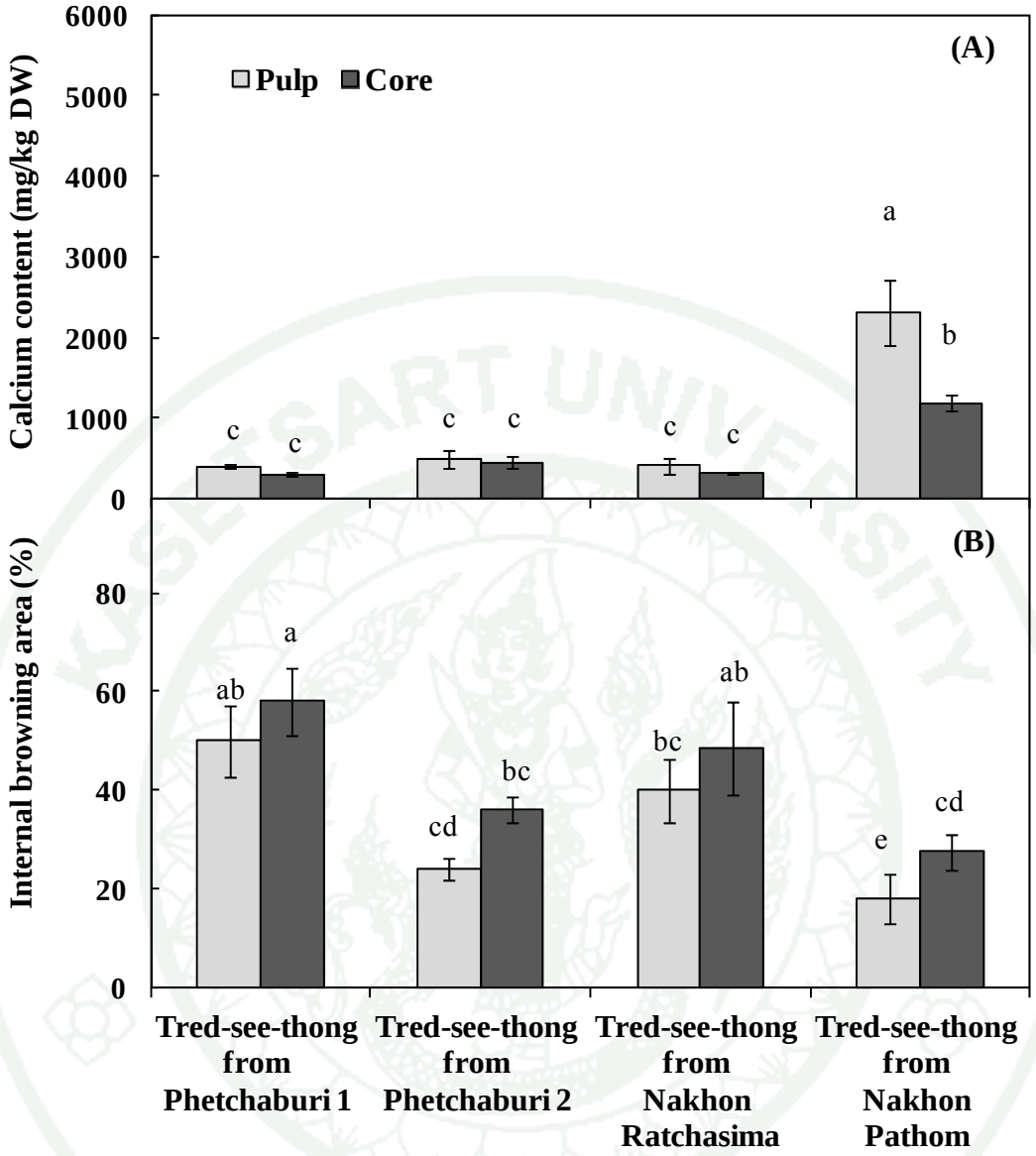

Figure 10 Total calcium contents before storage (A) and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C (B) in the pulp and the core of Trad-see-thong pineapples harvested from four different locations. Data are means ± SE of three replications (A) and ten replications (B). Bar with the different letters in the same row are significantly different at $P \leq 0.05$.

There was a significant negative correlation between total calcium content and IB of Trad-see-thong pineapple (r = -0.699, $P \leq 0.01$), as shown in Figure 11.

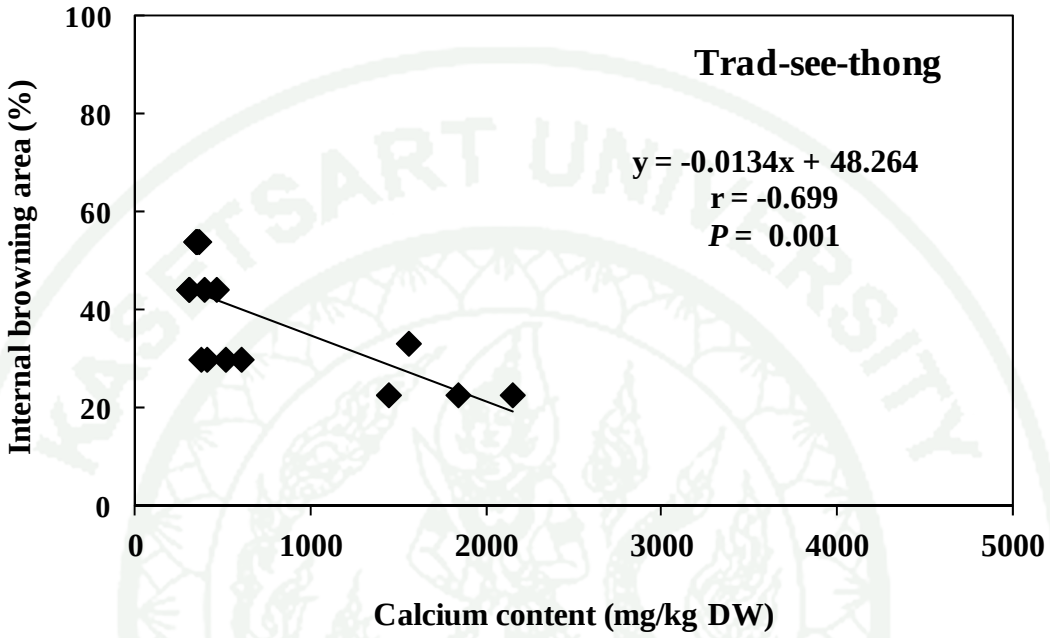

Figure 11 Correlation between total calcium content before storage and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong pineapple.

1.2 Effect of pre-harvest calcium applications on IB of Trad-see-thong pineapple

1.2.1 Effect of pre-harvest foliar calcium application on IB

Total calcium content in the pulp of foliar treated fruit was approximately 360 mg/kg DW, significantly higher than that in the control fruit which had less than 230 mg/kg DW, while the total calcium content in the core of foliar treated and control fruit were not significantly different (P>0.05) (Figure 12A). After storage at 10 °C for 21 days followed by 1 day at 25 °C, IB in the pulp of foliar treated fruit (20% IB of the pulp area) was significantly lower than that in the control fruit (31% IB of the pulp area), while IB in the core of foliar treated fruit and control fruit were not significantly different (44% of the core area in foliar treated fruit and 47% IB of core area in control fruit) (P>0.05) (Figure 12B).

1.2.2 Effect of pre-harvest soil calcium application on IB

Total calcium content in the pulp was highest (approximately 920 mg/kg DW) in 200 kg/ha CaO treated fruit, followed by 100 kg/ha CaO treated fruit (approximately 750 mg/kg DW) and was significantly the lowest in the control fruit (approximately 400 mg/kg DW) (Figure 13A). In the core, total calcium contents were not significantly different between the treated and control fruit (approximately 320-480 mg/kg DW) (P>0.05). After storage at 10 °C for 21 days followed by 1 day at 25 °C, IB symptoms in the pulp were not significantly different between the treated and control fruit, while IB symptom in the core was significantly lower in 200 kg/ha CaO treated fruit (28 % IB of the core area) than those in100 kg/ha CaO treated fruit (37% IB or the core area) and control fruit (48% IB of the core area) (Figure 13B).

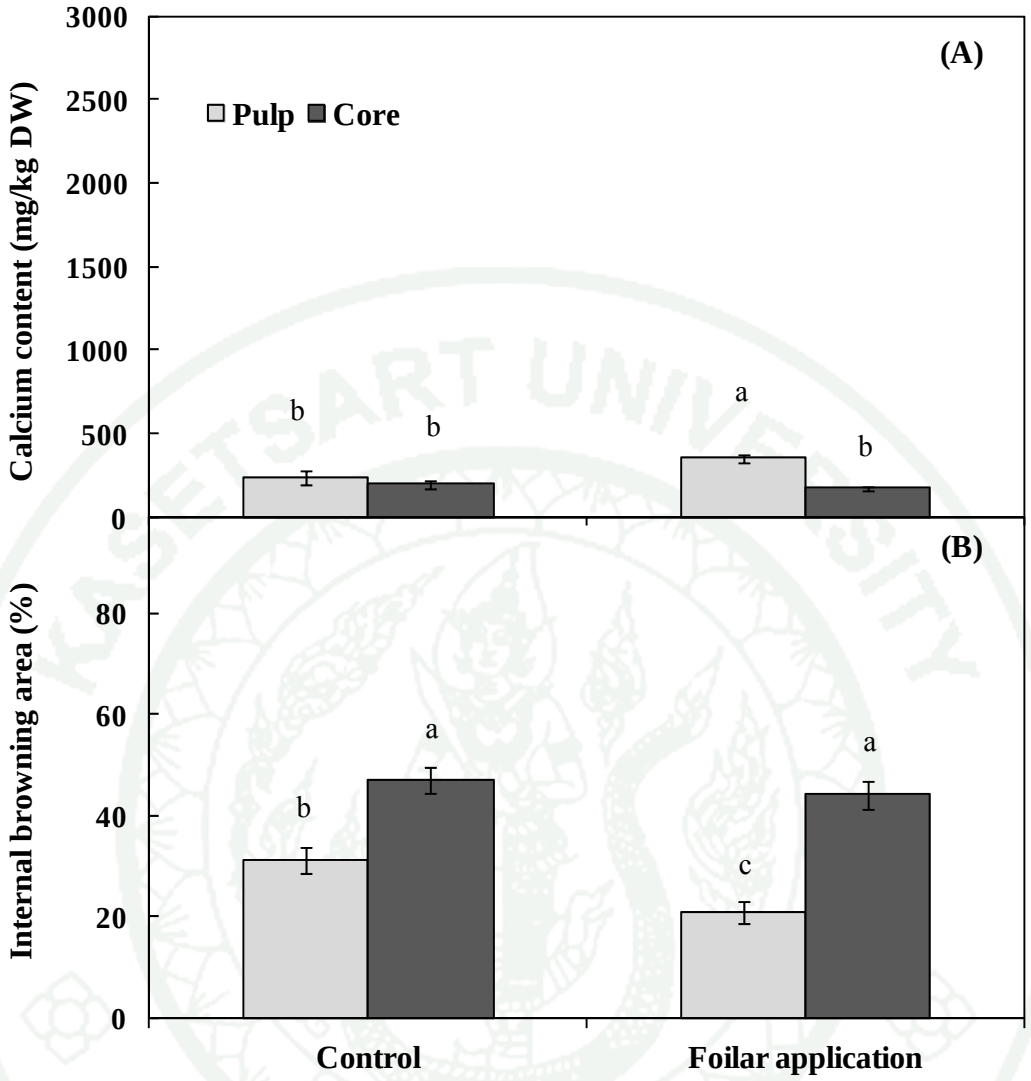

Figure 12 Total calcium contents at harvest (A) and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C (B) in the pulp and the core of Trad-see-thong pineapples after foliar calcium application. Data are means ± SE of three replications (A) and ten replications (B). Bar with the different letters in the same row are significantly different at $P \leq 0.05$.

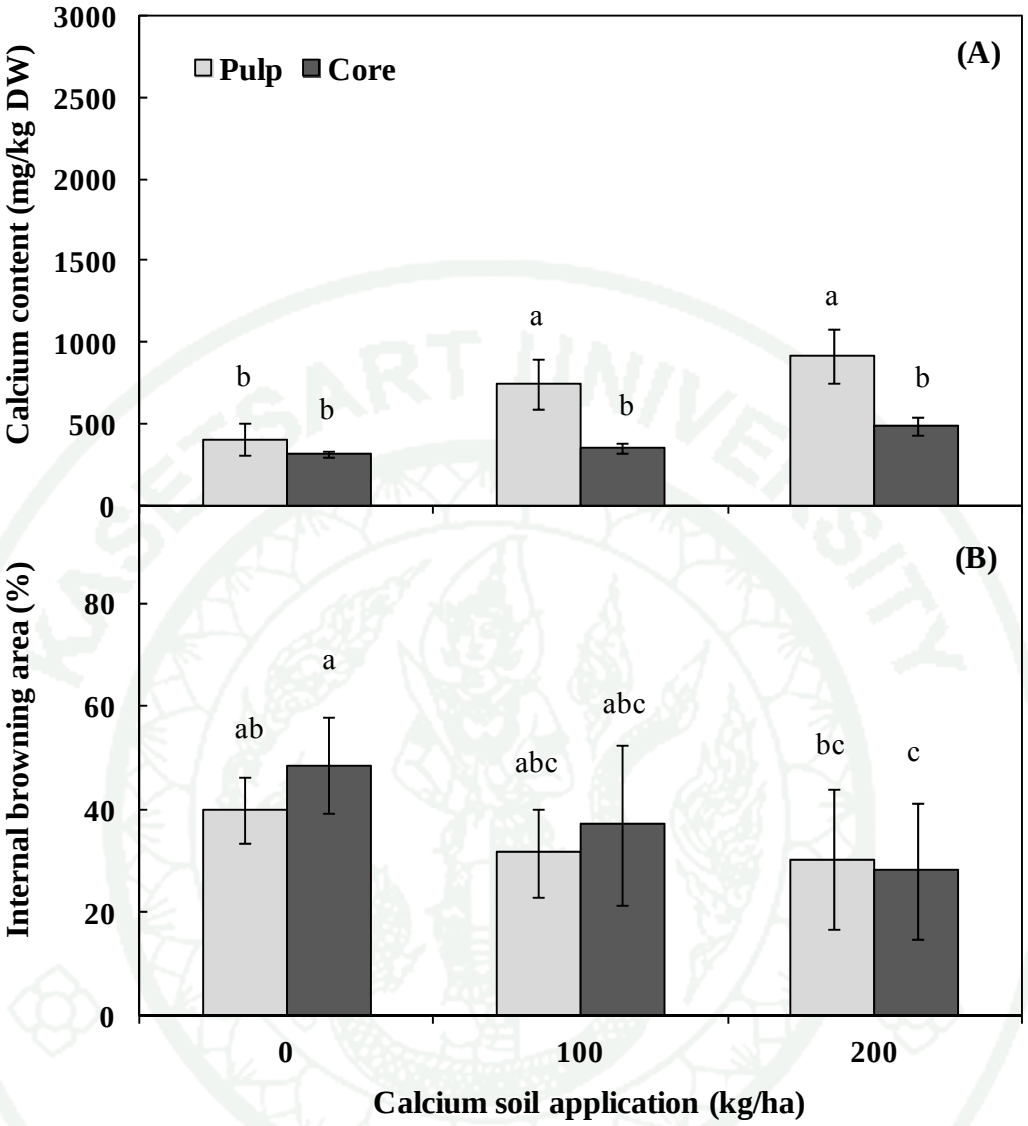

Figure 13 Total calcium contents at harvest (A) and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C (B) in the pulp and the core of Trad-see-thong pineapples after soil calcium applications. Data are means ± SE of three replications (A) and ten replications (B). Bar with the different letters in the same row are significantly different at $P \leq 0.05$.

1.2.3 Effect of pre-harvest calcium application on IB

Total calcium contents in the pulp (1,300-2,100 mg/kg DW) were higher than in the core (700-1,400 mg/kg DW) in all treatments. Total calcium contents in the pulp and the core were highest in the combined foliar and soil treated fruit (more than 2,100 and 1,400 mg/kg DW in the pulp and the core, respectively), followed by the soil treated fruit (less than 1,700 and 1,100 mg/kg DW in the pulp and the core, respectively) and the foliar treated fruit (less than 1,500 and 900 mg/kg DW in the pulp and the core, respectively) and was the lowest in the control fruit (less than 1,400 and 800 mg/kg DW in the pulp and the core, respectively). However, the calcium contents were not all significantly different (P>0.05) (Figure 14A).

After storage at 10 °C for 21 days followed by 1 day at 25 °C, in the pulp, IB symptom was significantly lower in combined foliar and soil treated fruit (11% IB of the pulp area) than in the soil treated fruit (19% IB of the pulp area), foliar treated fruit (22% IB of the pulp area) and the control fruit (26% IB of the pulp area). In the core, IB symptom was significantly lowest in combined foliar and soil treated fruit (7% IB of the core area), follow by soil treated fruit (14% IB of the core area) or foliar treated fruit (14% IB of the core area) and was highest in control fruit (27% IB of the core area) (Figure 14B). Combined foliar and soil treated fruit had a 57% and 71% reduction in IB in the pulp and the core, respectively. Foliar treated fruit and soil treated fruit had slightly less IB than the control fruit, with a 14% and 24% reduction in IB in the pulp and a 47% and 47% reduction in the core, respectively.

After storage at 10 °C for 21 days followed by 1 day at 25 °C, the pulp color in the control fruit became more yellow, while that in the calcium treated fruit remained pale yellow (Figure 15).

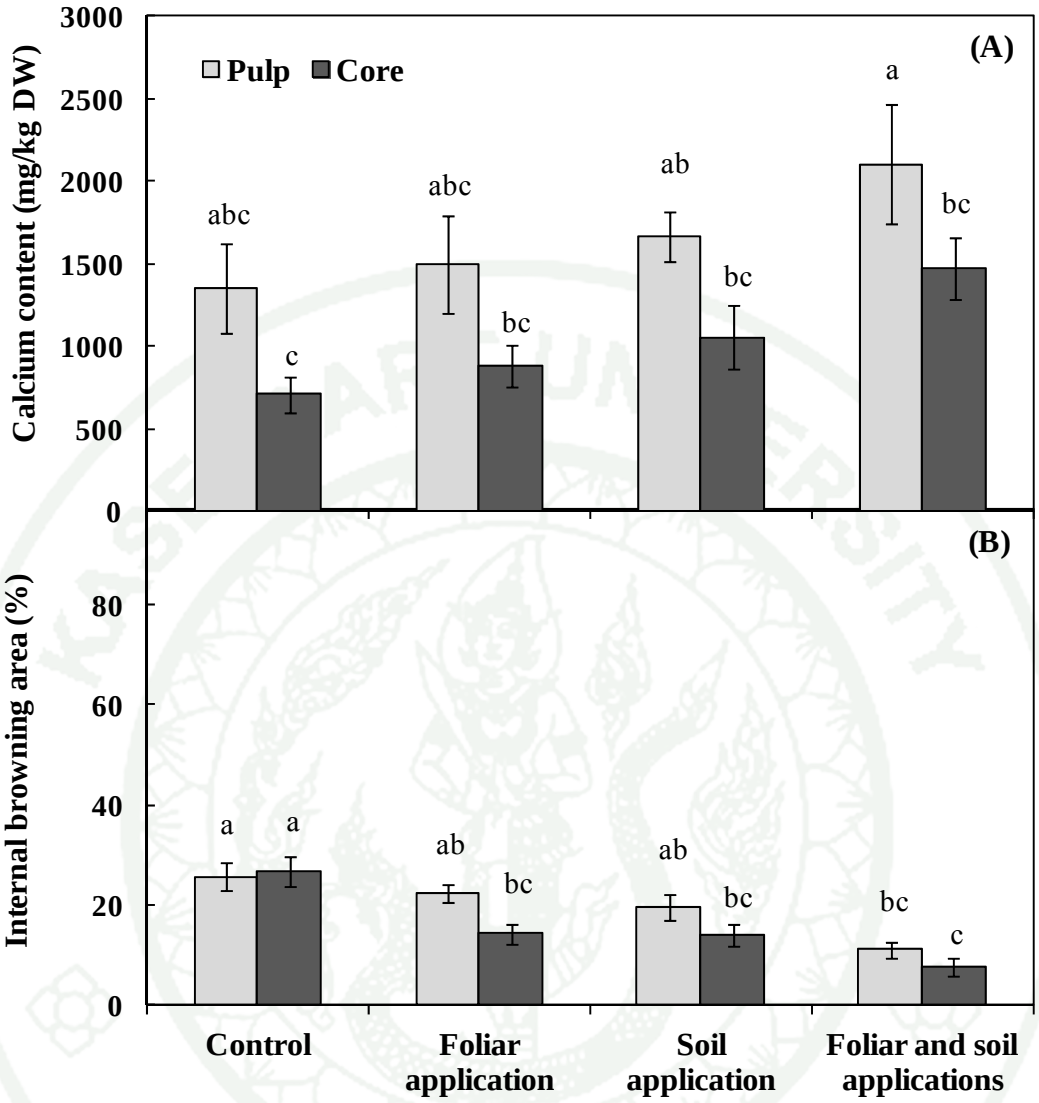

Figure 14 Total calcium contents at harvest (A) and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C (B) in the pulp and the core of Trad-see-thong pineapples after different pre-harvest calcium applications. Data are means ± SE of five replications (A) and fifty replications (B). Bar with the different letters in the same row are significantly different at $P \leq 0.05$.

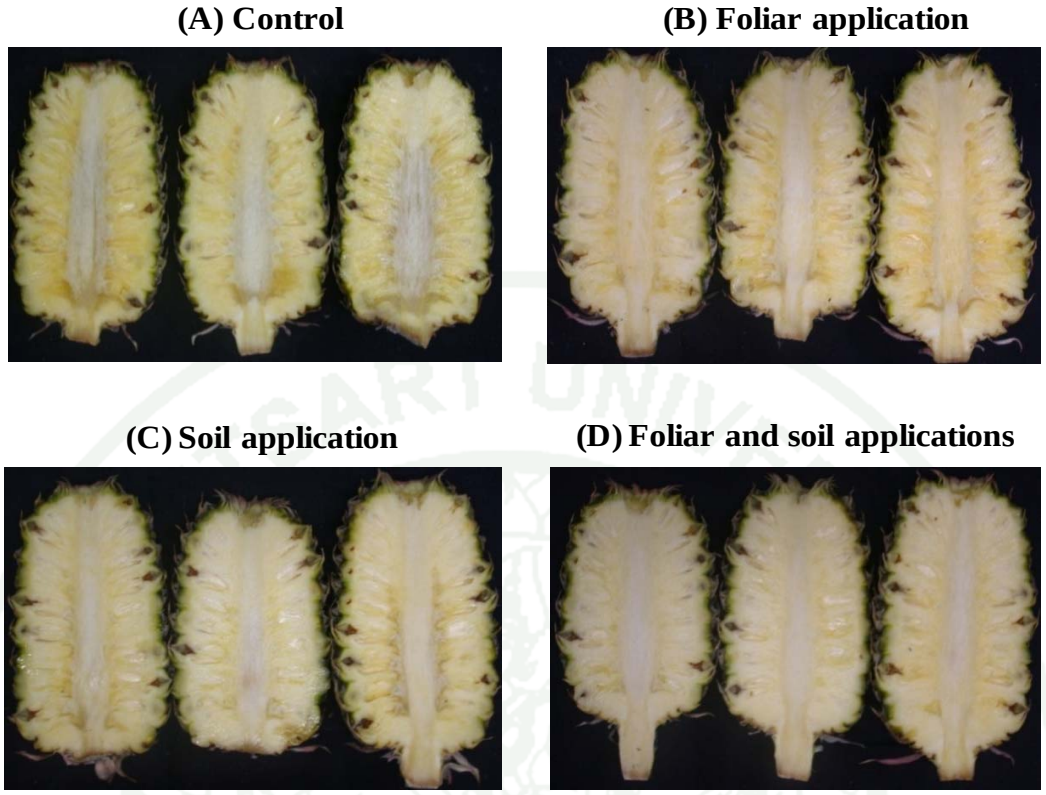

Figure 15 Internal browning after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong pineapples (A) and fruit treated with calcium by foliar (B), soil (C) and foliar and soil (D) applications.

1.3 Effect of post-harvest calcium application on IB of Trad-see-thong pineapple

1.3.1 Fixed submersion time in different calcium chloride solutions

Postharvest calcium applications by immersing pineapple fruit stem in calcium chloride ($CaCl_2$) solution for 18 hours significantly increased total calcium contents in the pulp from approximately 260 mg/kg DW in the control fruit to approximately 790 mg/kg DW in 1% $CaCl_2$ treated fruit. Increasing the concentration of $CaCl_2$ by 2 and 4 times only increased the calcium content s in pulp further by 1.2 and 1.5 times, respectively. The calcium content in the core also significantly increased from 310 mg/kg DW in the control to 1,050 mg/kg DW in 1% $CaCl_2$ treated fruit. By increasing $CaCl_2$ concentration by 2 and 4 times increased the calcium contents in the core by 1.8 and 3 times, respectively (Figure 16A). Total calcium content in the core was higher than that in the pulp. These postharvest calcium applications by fruit stem submersion in 1, 2 and 4% $CaCl_2$ solution reduced IB symptoms by 35, 65 and 80% in the pulp and 33, 56, and 76% in the core, respectively. However, IB symptoms in the pulp in this experiment were slight IB (2-11% IB of the pulp area) and were lower than those in the core (6-26 % IB of the core area). In the control fruit, the symptom was observed at only 10% of the pulp area and 26% of the core area. The effect of postharvest calcium treatment on IB in the core followed the same trend found in the pulp (Figure 16B).

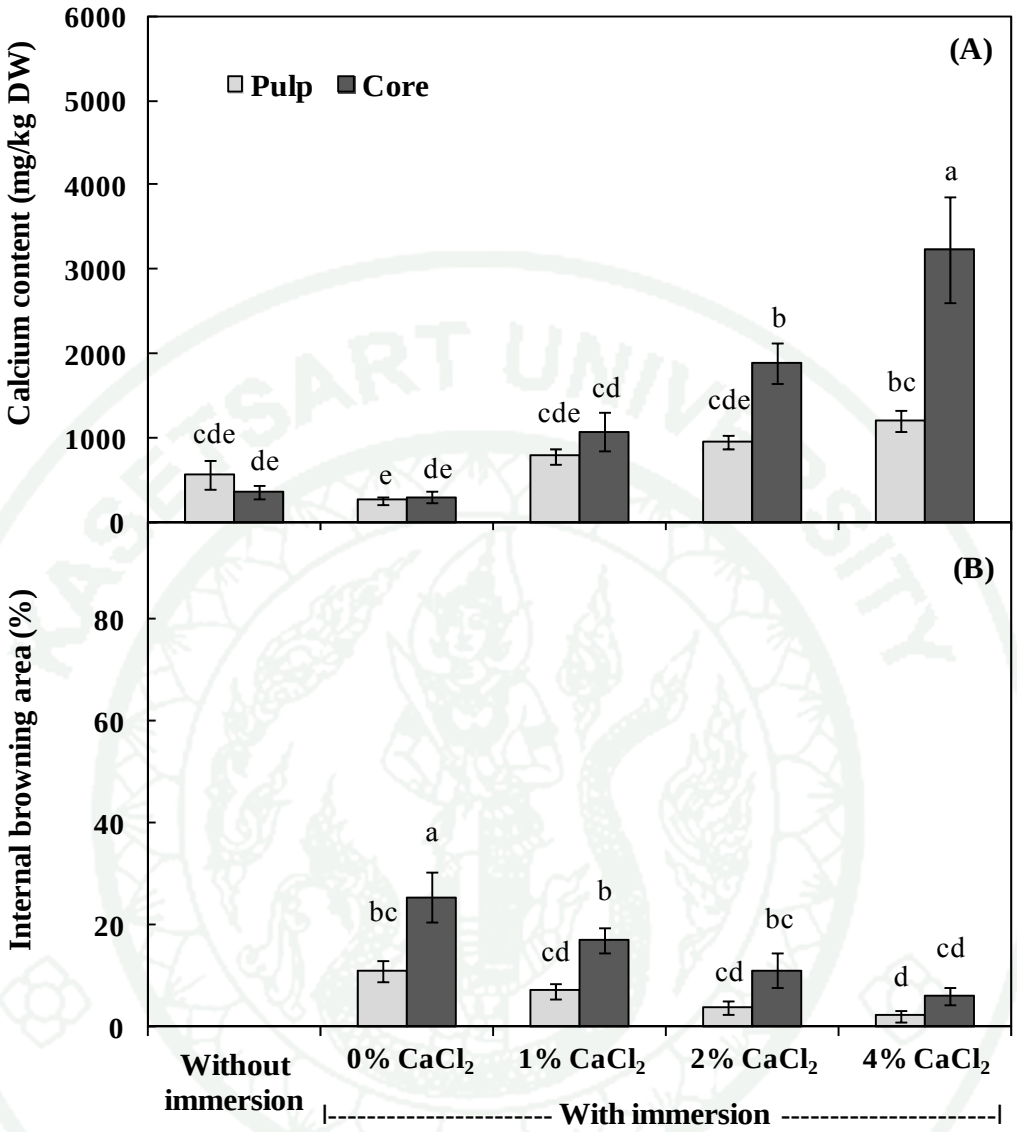

Figure 16 Total calcium contents without and with immersion (A) and internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C (B) in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0, 1, 2 and 4 % $CaCl_2$ solutions for 18 hours. Data are means ± SE of three replications (A) and ten replications (B). Bar with the different letters in the same row are significantly different at $P \leq 0.05$.

However, the 2 and 4 % $CaCl_2$ application caused a dark brown area in the fruit stem, which extended 2.5 centimeters into the core (Figure 17).

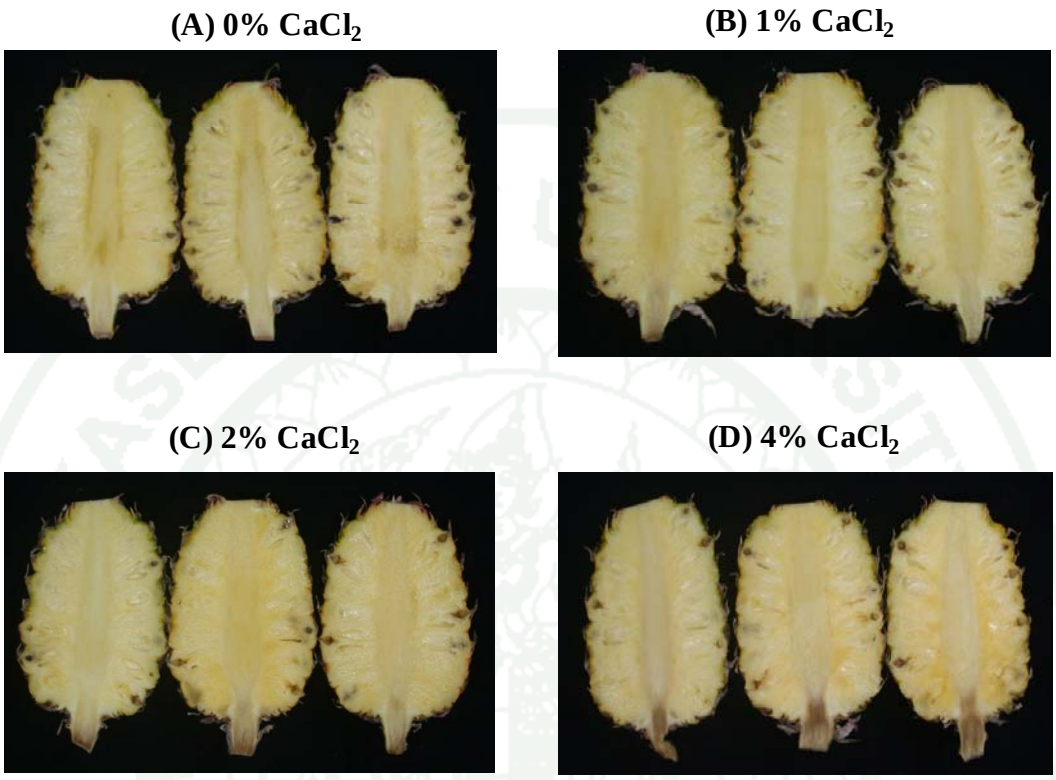

Figure 17 Internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong pineapples; stem submersion in 0% $CaCl_2$ as control (A), 1% $CaCl_2$ (B), 2% $CaCl_2$ (C), 4% $CaCl_2$ (D) solutions for 18 hours.

Furthermore, similar repeated experiment of postharvest calcium applications by fruit stem dipped in 1, 2 and 4% $CaCl_2$ solution for 18 hour could not reduce IB symptoms in Trad-see-thong pineapple (Data shown in the Appendix Table 7).

1.3.2 Fixed submersion time in calcium chloride solutions

In the control fruit, approximately 45% of the pulp area and 47 % of the core area developed IB symptom after 21 days storage. With increased $CaCl_2$ concentrations from 0 to 2%, IB of the pulp area increased, but was not significantly different (P>0.05). However, IB in the core shown no change in 2% $CaCl_2$ treated fruit as compared to the control fruit (Figure 18).

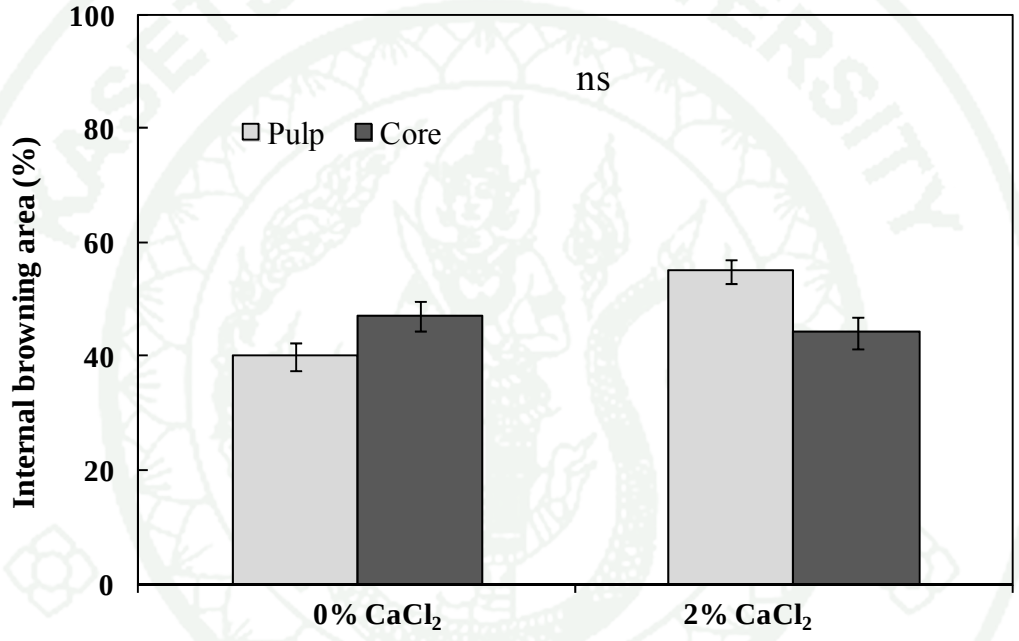

Figure 18 Internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0 and 2 % $CaCl_2$ solutions for 18 hours. Data are means ± SE of ten replications. Ns is non-significantly different (P>0.05).

The degree of IB in pineapple after submersion in 0 and 2 % $CaCl_2$ solutions for 18 hours and stored at 10 °C for 21 days followed by 1 day at 25 °C are shown in Figure 19. Additionally, the 2 % $CaCl_2$ application caused a dark brown area in the fruit stem, which extended 2.5 centimeters into the core.

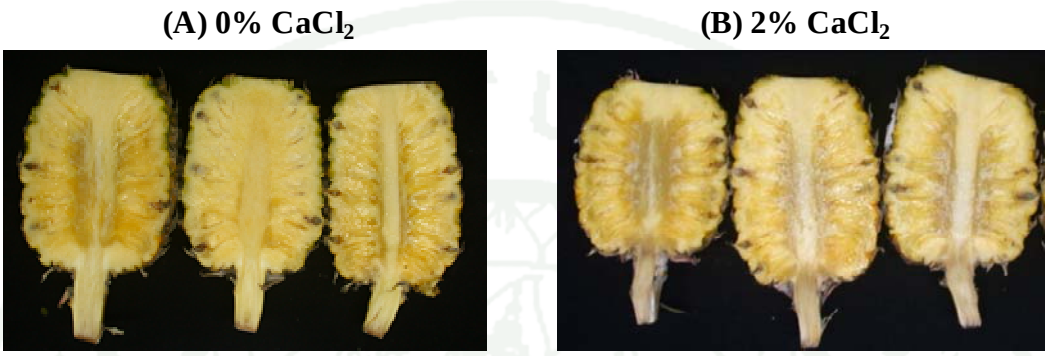

Figure 19 Internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong pineapples; stem submersion in 0% $CaCl_2$ as control (A) and 2 % $CaCl_2$ (B) solutions for 18 hours.

Furthermore, three similar repeated experiments of postharvest calcium applications by fruit stem submersion in 2 % $CaCl_2$ solution for 18 hour could not reduce IB symptom in Trad-see-thong pineapple (Data shown in the Appendix Table 9-11).

1.3.3 Different submersion duration in different calcium concentrations

In the control fruit, approximately 45-65% of the cut surface area developed IB symptom after 21 days storage. With increased $CaCl_2$ concentrations, IB of 1 and 1.5 % $CaCl_2$ treated fruits were significantly higher than that in control. When the submersion duration was increased from 24 hours to 48 and 72 hours, IB significantly decreased. In submersion duration of 72 hours, IB was the lowest, followed by submersion durations of 24 and 48 hours. However, there was no interaction between concentration and submersion duration, as shown in Table 1.

Table 1 Internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong pineapples; stem submersion in 0 (Control), 0.5, 1 and 1.5% $CaCl_2$ solutions for 24, 48 and 72 hours.

Treatment	Hours	Internal browning area (%)[1]
0% $CaCl_2$	24	50.60±7.46
	48	63.33±2.29e
	72	44.93±7.73
0.5% $CaCl_2$	24	51.73±8.30
	48	68.20±4.19
	72	49.36±9.73
1% $CaCl_2$	24	76.30±5.05
	48	72.52±4.77
	72	53.08±8.89
1.5% $CaCl_2$	24	71.74±7.51
	48	66.69±7.95
	72	54.70±7.00
Concentration		*
Time		**
Concentration × Time		ns
0% $CaCl_2$		b
0.5% $CaCl_2$		ab
1% $CaCl_2$		a
1.5% $CaCl_2$		a
24 hours		a
48 hours		a
72 hours		b
CV (%)		34.20

[1] Mean±SE value and the different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

***** = significantly different at $0.01 < P \leq 0.05$

****** = significantly different at $P \leq 0.01$

The IB symptom in pineapple after submersion in different calcium concentrations and for different duration and stored at 10 °C for 21 days followed by 1 day at 25 °C are shown in Figure 20.

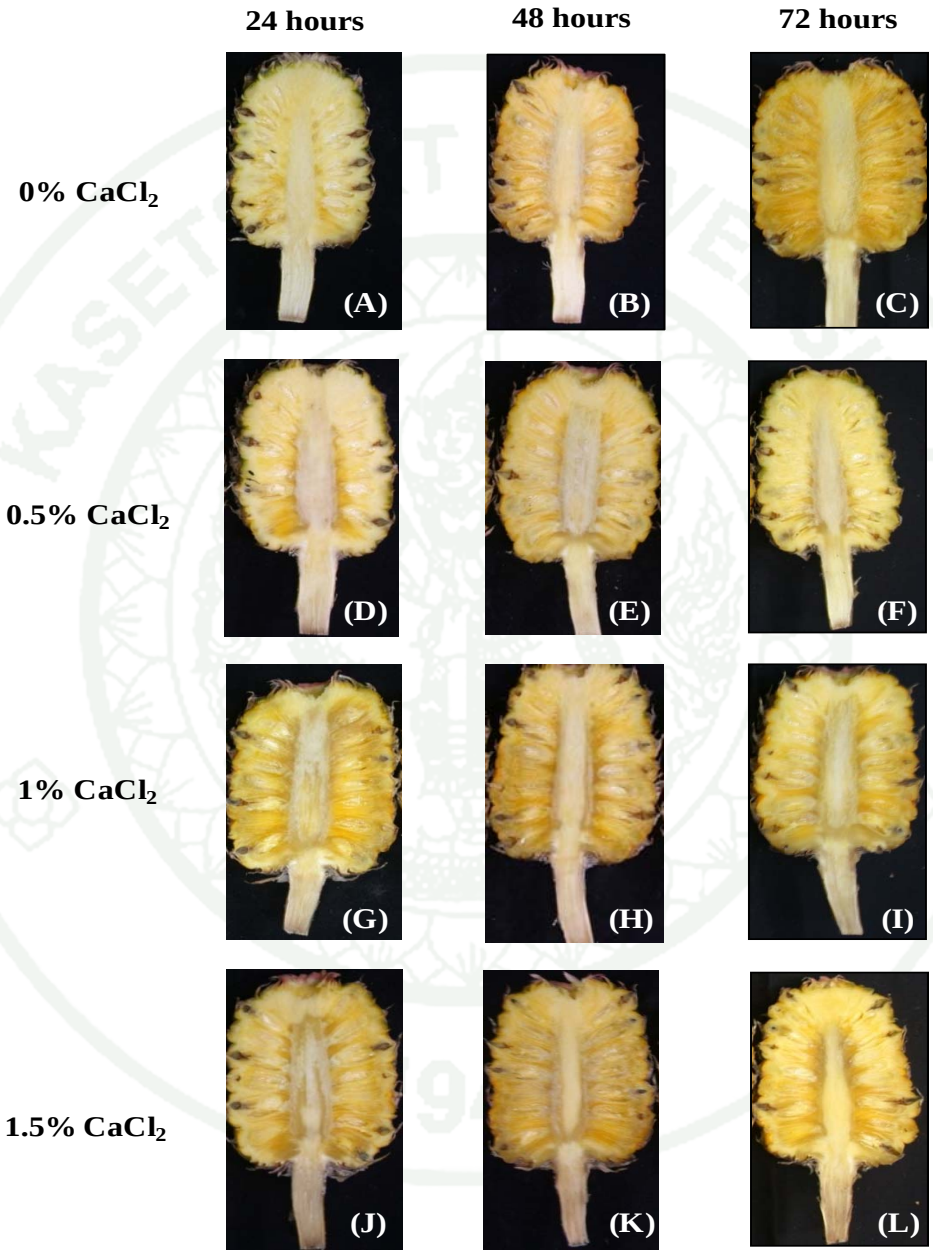

Figure 20 Internal browning area after storage at 10 °C for 21 days followed by 1 day at 25 °C of Trad-see-thong pineapples; stem submersion in 0% $CaCl_2$ as control (A, B and C), 0.5% $CaCl_2$ (D, E and F), 1% $CaCl_2$ (G, H and I), 1.5% $CaCl_2$ (J, K and L) solutions for 24, 48 and 72 hours.

2. The role of endogenous GA and ABA in IB development in pineapple

2.1 The changes of endogenous GA and ABA concentrations and IB of pineapple

Pineapples cvs. Trad-see-thong and Pattavia were assembled into 3 groups; (1) the fruit were stored at 10 °C (85±5% RH) for 21 days (10 °C), (2) the fruit were stored at 10 °C (85±5% RH) but were subjected to 25 ºC for one day on either the 7th, 14th, 21st day (10 °C + 1 d 25 °C), (3) the fruit were stored at 25 °C (85±5% RH) for 21 days (25 °C).

1) Internal browning area

IB was not found in Trad-see-thong and Pattavia pineapple stored at 25 °C for 21 days. In both these pineapple cultivars, the percentage of fruits exhibiting IB was significantly higher in 10 °C + 1 d 25 °C-treated fruit compared to the 10 °C-treated fruit (Figure 21A and 21B). At day 21, IB of 10 °C + 1 d 25 °C-treated fruit were approximately 50% and 22% in Trad-see-thong and Pattavia, respectively, and IB of 10 °C-treated fruit were 40% and 10% in Trad-see-thong and Pattavia, respectively. Trad-see-thong fruit was more sensitive to low temperatures than Pattavia fruit. At 10 °C, Trad-see-thong developed IB after day 7 and rapidly increased after day 14, while IB in Pattavia was not observed until day 14. IB in Trad-see-thong at 10 °C for 21 days + 1 d 25 °C was twice as severe as that in Pattavia.

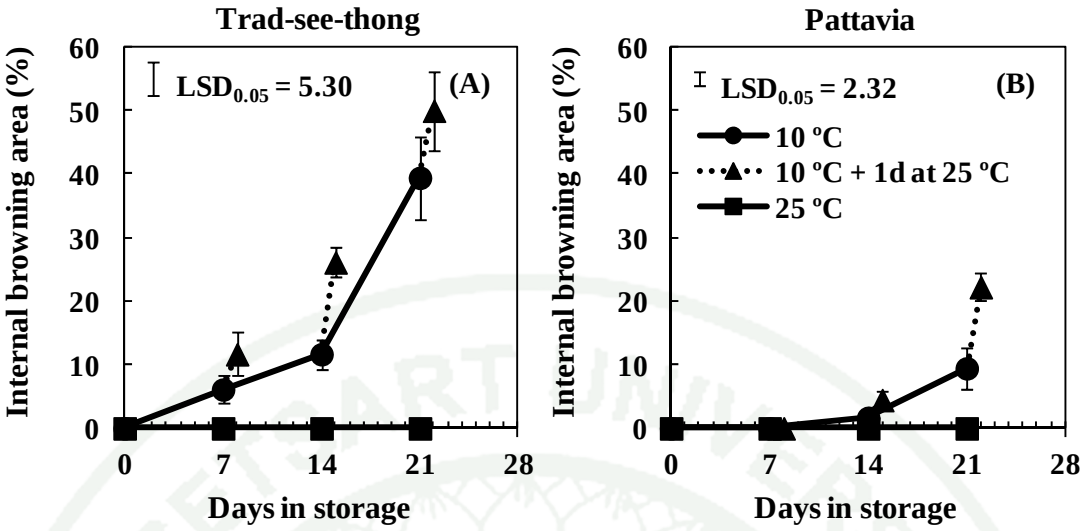

Figure 21 Internal browning area of Trad-see-thong (A) and Pattavia (B) pineapples during storage at 10 °C, 10 °C + 1 d 25 °C, and 25 °C. Data are mean ± SE of ten replications.

2) PPO activity

In both pineapple cultivars, PPO activity was significantly higher in the 10 °C-treated fruit compared to the 25 °C-treated fruit (Figure 22A and 22B). In Trad-see-thong, PPO activity was significantly highest in the 10 °C + 1 d 25 °C-treated fruit, followed by fruit stored at 10 °C, and was lowest in the fruit stored at 25 °C. However, there were no significant differences in Pattavia between the 10 °C + 1 d 25 °C and 10 °C conditions (P>0.05). PPO activities were higher in Trad-see-thong than in Pattavia. At 10 °C, PPO activities in Trad-see-thong and Pattavia rapidly increased from approximately 0.42 to 1.23 units/mg protein and from 0.42 to 0.83 units/mg protein during storage, respectively.

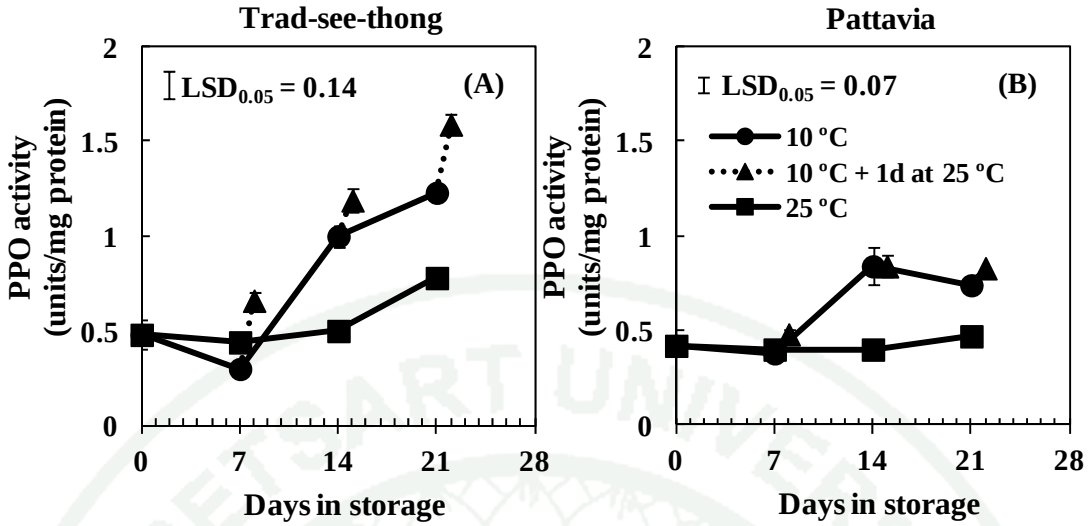

Figure 22 PPO activities of Trad-see-thong (A) and Pattavia (B) pineapples during storage at 10 °C, 10 °C + 1 d 25 °C, and 25 °C. Data are mean ± SE of three replications.

3) Endogenous GA_1, GA_3 and GA_4 concentrations

In both pineapple cultivars, the endogenous GA_1, GA_3 and GA_4 concentrations were not significantly different between fruit stored at 10 °C and fruit stored at 25 °C (P>0.05), except for GA_1 in Trad-see-thong at day 14 (Figure 23A-23F). After the transfer from 10 °C to 25 °C, the endogenous GA_1 concentrations were significantly increased at day 21 in both cultivars (from 0.25 to 1.80 µmol/kg FW in Trad-see-thong and from 0.11 to 1.65 µmol/kg FW in Pattavia) (Figure 23A and 23B). The endogenous GA_3 concentration in fruit after the transfer from 10 °C to 25 °C was significantly increased at day 14 (from 1.33 to 3.48 µmol/kg FW) and day 21 (from 1.02 to 3.41 µmol/kg FW) in Trad-see-thong (Figure 23C). While endogenous GA_3 concentrations in Pattavia were not significantly different between 10 °C + 1 d 25 °C-treated fruit and other treated fruit (P>0.05) (Figure 23D). The endogenous GA_4 concentration in fruit after transfer from 10 °C to 25 °C was significantly increased at day 7 (from 0.67 to 2.18 µmol/kg FW), day 14 (from 0.49 to 1.75 µmol/kg FW) and day 21 (from 0.75 to 1.06 µmol/kg FW) in Trad-see-thong (Figure 23E) and was significantly increased at day 7 (from 0.50 to 1.30 µmol/kg FW) in Pattavia (Figure

23F). The endogenous GA_3 concentrations were higher than endogenous GA_1 and GA_4 concentrations in both cultivars.

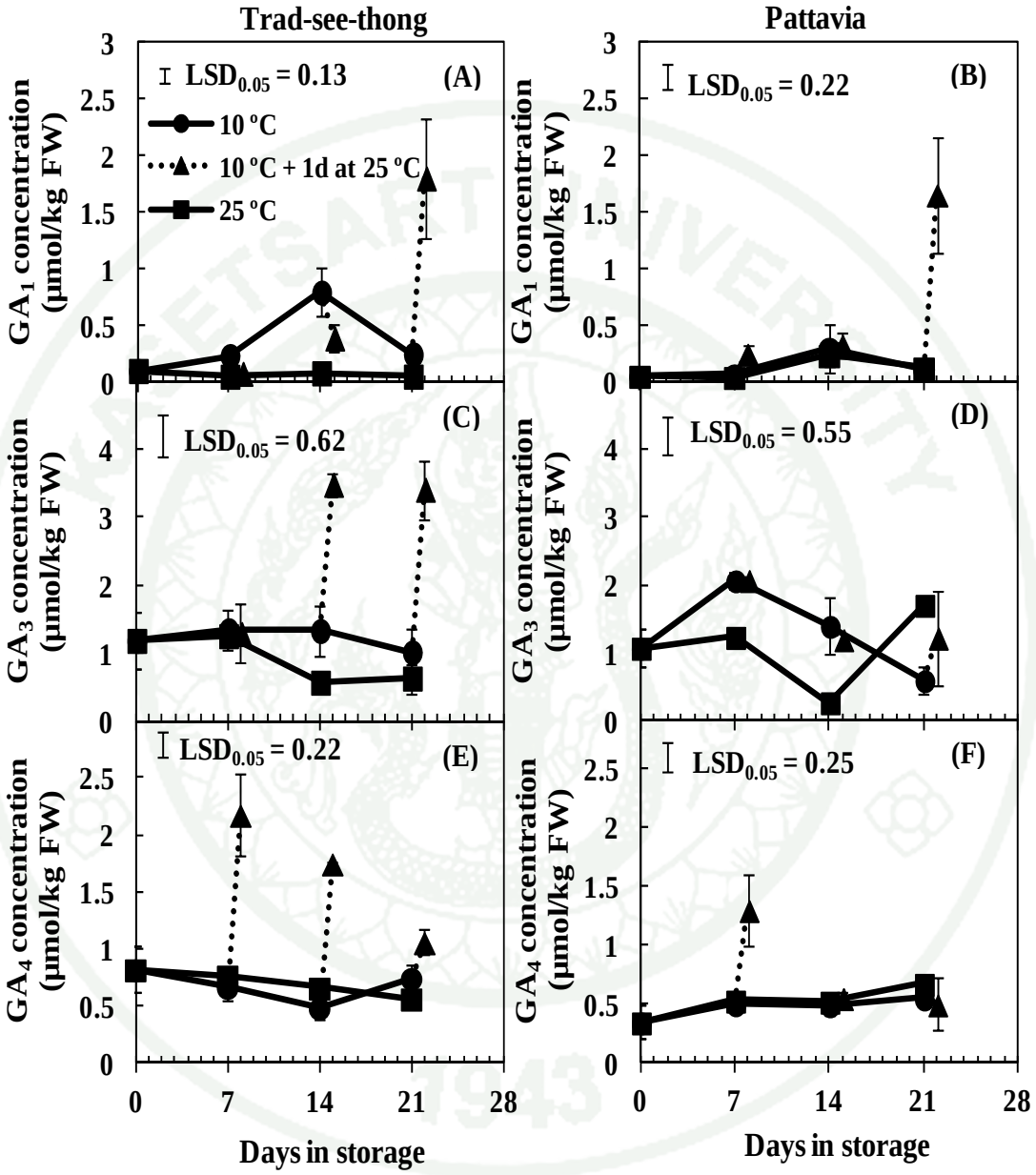

Figure 23 Endogenous GA_1, GA_3 and GA_4 concentrations in Trad-see-thong (A, C, E) and Pattavia (B, D, F) pineapples during storage at 10 °C, 10 °C + 1 d 25 °C, and 25 °C. Data are mean ± SE of three replications.

Total GA ($GA_1 + GA_3 + GA_4$) concentrations in Trad-see-thong was not significantly different between fruit stored at 10 °C and fruit stored at 25 °C (P>0.05), while total GA in Pattavia was significantly increased in fruit stored at 10 °C compared to fruit stored at 25 °C. After the transfer from 10 °C to 25 °C, total GA concentrations significantly increased in both cultivars, but were higher in Trad-see-thong than in Pattavia. At day 21, total GA concentrations in Trad-see-thong and Pattavia were increased from 0.69 to 2.16 mg/kg FW and from 0.75 to 1.61 mg/kg FW after the transfer from 10 °C to 25 °C, respectively (Figure 24A and 24B).

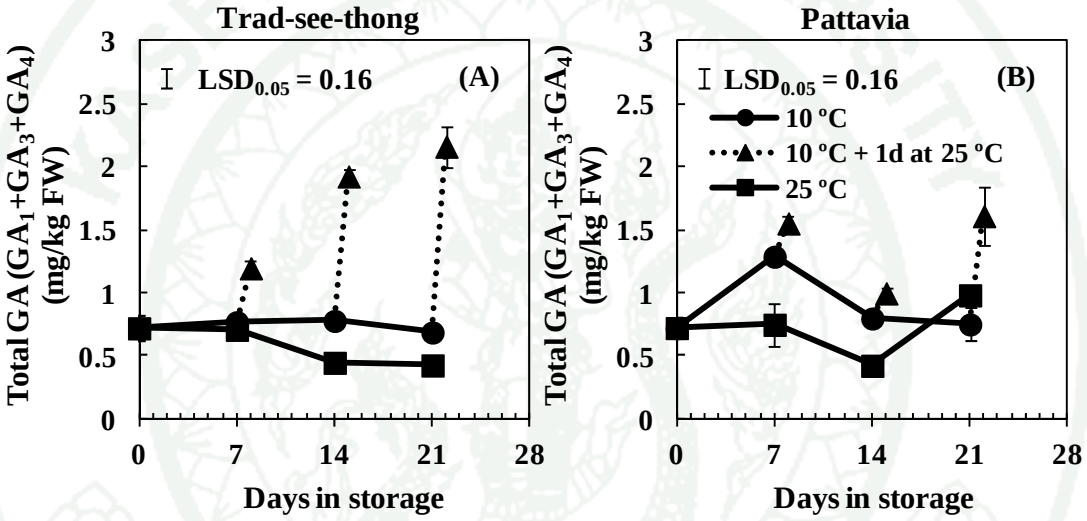

Figure 24 Total GA (endogenous $GA_1 + GA_3 + GA_4$) concentrations in Trad-see-thong (A) and Pattavia (B) pineapples during storage at 10 °C, 10 °C + 1 d 25 °C, and 25 °C. Data are mean ± SE of three replications.

4) Endogenous ABA concentrations

In both pineapple cultivars, the endogenous ABA concentrations were significantly increased in fruit stored at 10 °C compared to fruit stored at 25 °C. In 10 °C + 1 d 25 °C-treated fruit, the endogenous ABA concentrations were significantly decreased in both cultivars compared to fruit stored at 10 °C. The endogenous ABA concentrations did not show a significant difference between the 10 °C + 1 d 25 °C and the 25 °C conditions (P>0.05), except at day 21 in Trad-see-

thong. The increase in endogenous ABA concentrations in Trad-see-thong was higher and occurred earlier than that in Pattavia at 10 °C (Figure 25A and 25B).

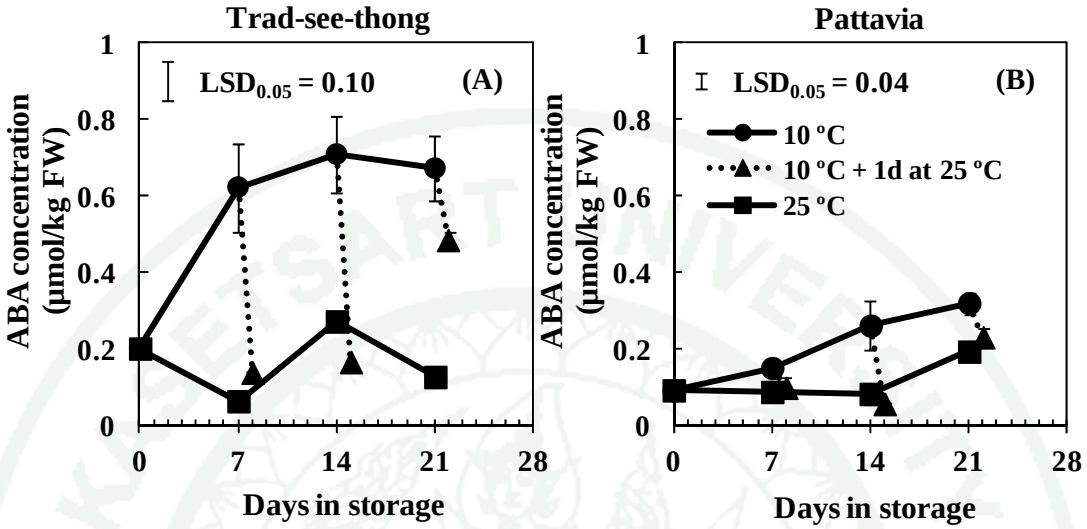

Figure 25 Endogenous ABA concentrations in Trad-see-thong (A) and Pattavia (B) pineapples during storage at 10 °C, 10 °C + 1 d 25 °C, and 25 °C. Data are mean ± SE of three replications.

2.2 Effect of GA_3 application on IB during storage at 25 °C

1) Internal browning area

In both pineapple cultivars, GA_3 application significantly increased the percentage of IB symptoms at days 14 and 21 during storage at 25 °C, while IB was not found in the control fruit (Figure 26A and 26B). In Trad-see-thong, IB symptoms in fruit treated with GA_3 at days 14 and 21 were 19.5 and 76.5%, respectively (Figure 26A). In Pattavia, IB symptoms in fruit treated with GA_3 at 14 and 21 day were 20 and 41.5%, respectively (Figure 26B). IB symptom at day 21 in Trad-see-thong that had been treated with GA_3 was higher than that in Pattavia.

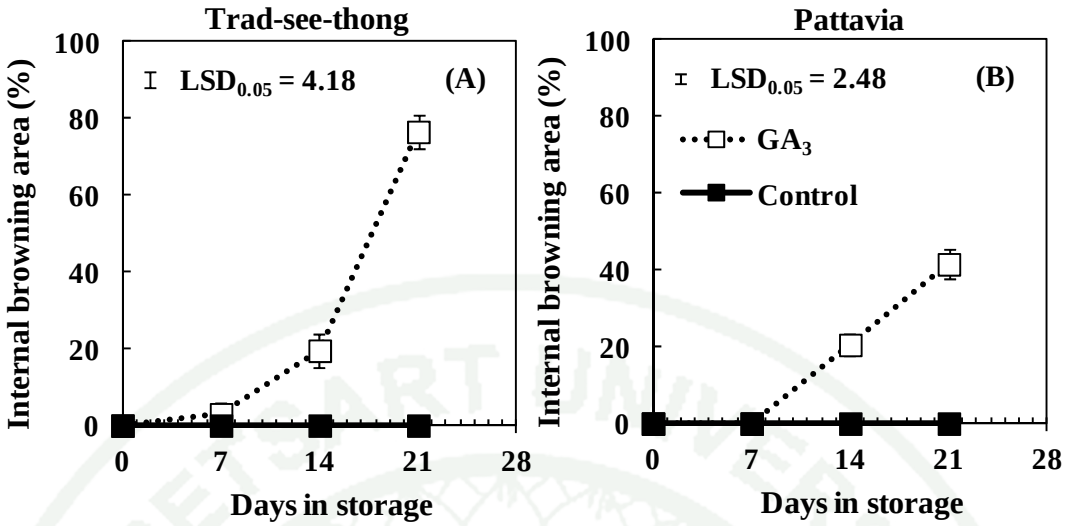

Figure 26 Internal browning area of Trad-see-thong (A) and Pattavia (B) pineapples treated with GA_3 and stored at 25 °C for 21 days. Data are mean ± SE of ten replications.

Additionally, the photographs of IB in both pineapple cultivars treated with GA_3 are shown in Figure 27.

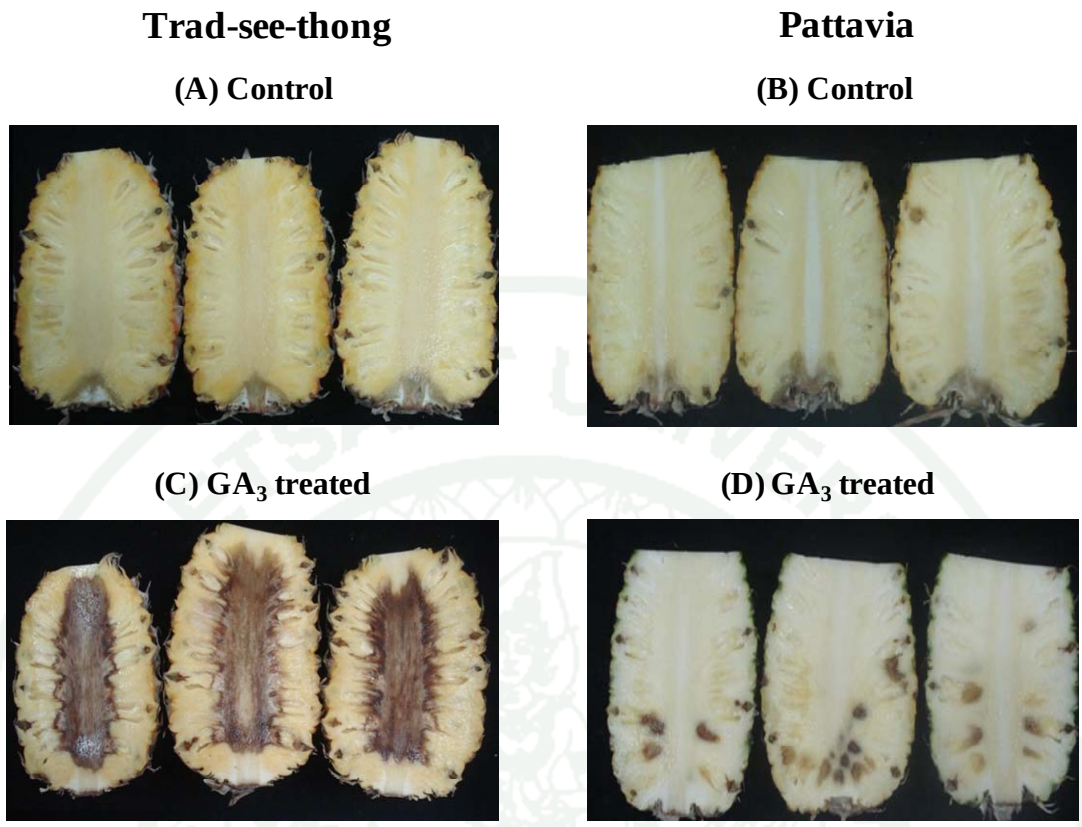

Figure 27 Internal browning of Trad-see-thong (A and C) and Pattavia (B and D) pineapples treated with GA_3 and stored at 25 °C for 21 days.

2) PPO activity

In both pineapple cultivars, GA_3 application significantly increased PPO activity at days 14 and 21 during storage at 25 °C (Figure 28A and 28B). In Trad-see-thong, PPO activities in fruit treated with GA_3 at days 14 and 21 were rapidly increased from 0.72 units/mg protein at day 0 to 1.59 and to 1.52 units/mg protein, respectively, while PPO activity in control fruit was slightly increased from 0.72 to 0.87 units/mg protein during storage at 25 °C (Figure 28A). In Pattavia, PPO activities in fruit treated with GA_3 rapidly increased at days 14 and 21 from 0.64 units/mg protein at day 0 to 0.94 and to 1.64 units/mg protein, respectively, while PPO activity in the control fruit slightly increased from 0.64 to 0.92 units/mg protein during storage at 25 °C (Figure 28B).

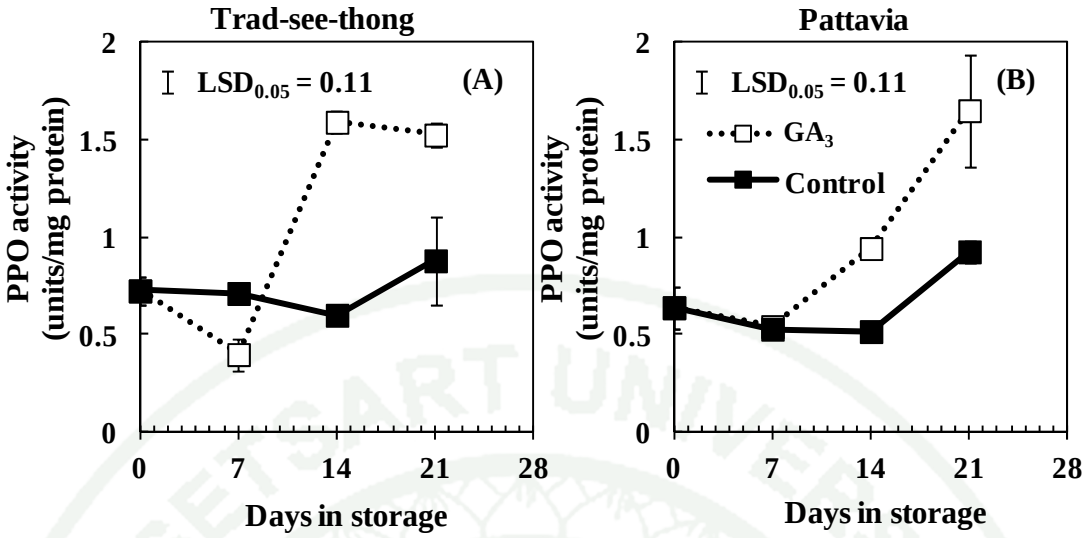

Figure 28 PPO activities of Trad-see-thong (A) and Pattavia (B) pineapples treated with GA_3 and stored at 25 °C for 21 days. Data are mean ± SE of three replications.

3) Endogenous ABA concentration

In Trad-see-thong, GA_3 application significantly increased the endogenous ABA concentration during storage at 25 °C (Figure 29A). Endogenous ABA concentration in fruit treated with GA_3 was slightly increased from day 0 to day 7 (from 0.20 to 0.24 µmol/kg FW) and rapidly increased from day 7 to day 14 (from 0.24 to 0.93 µmol/kg FW) and then rapidly decreased until day 21 (from 0.93 to 0.24 µmol/kg FW), while endogenous ABA concentration in control fruit fluctuated during storage at 25 °C. In Pattavia, the endogenous ABA concentration in fruit treated with GA_3 at days 7 and 14 was slightly increased from 0.09 µmol/kg FW at day 0 to 0.16 and to 0.18 µmol/kg FW, respectively, and then decreased to 0.03 µmol/kg FW at day 21 during storage at 25 °C (Figure 29B). The ABA concentration at day 14 in Trad-see-thong that had been treated with GA_3 was higher than that in Pattavia.

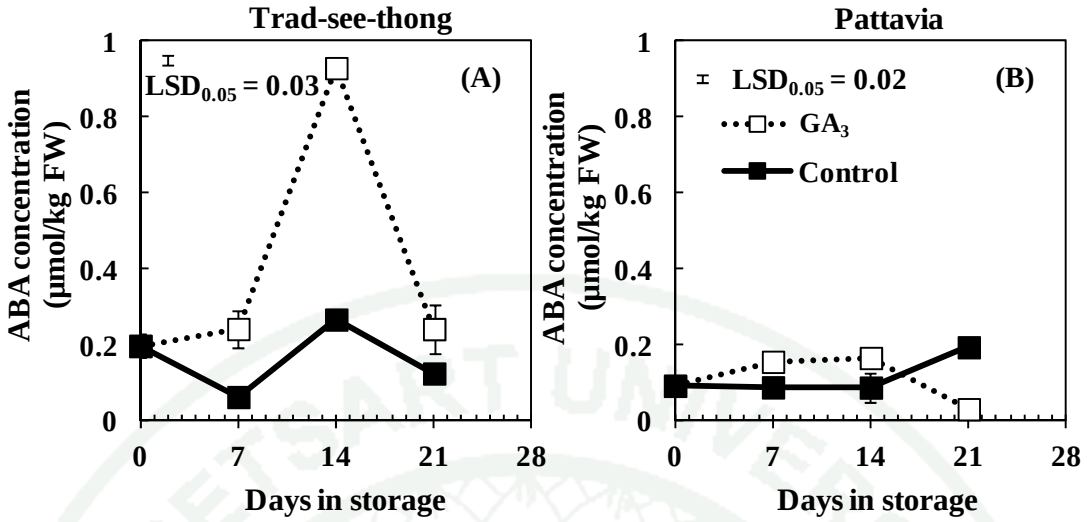

Figure 29 Endogenous ABA concentrations in Trad-see-thong (A) and Pattavia (B) pineapples treated with GA_3 and stored at 25 °C for 21 days. Data are mean ± SE of three replications.

2.3 Effect of GA_3 application on IB during storage at 10 °C

1) Internal browning area

In both pineapple cultivars, GA_3 application significantly reduced the percentage of IB symptoms at day 21 during storage at 10 °C, while IB in the control fruit increased after day 7 in Trad-see-thong and after days 14 in Pattavia (Figure 30A and 30B). In Trad-see-thong at day 21 during storage at 10 °C, IB symptoms in fruit treated with GA_3 were 10%, while IB symptom in control fruit was 39% (Figure 30A). In Pattavia at day 21 during storage at 10 °C, IB symptoms in fruit treated with GA_3 were 3%, while IB symptom in the control fruit was 9.4% (Figure 30B).

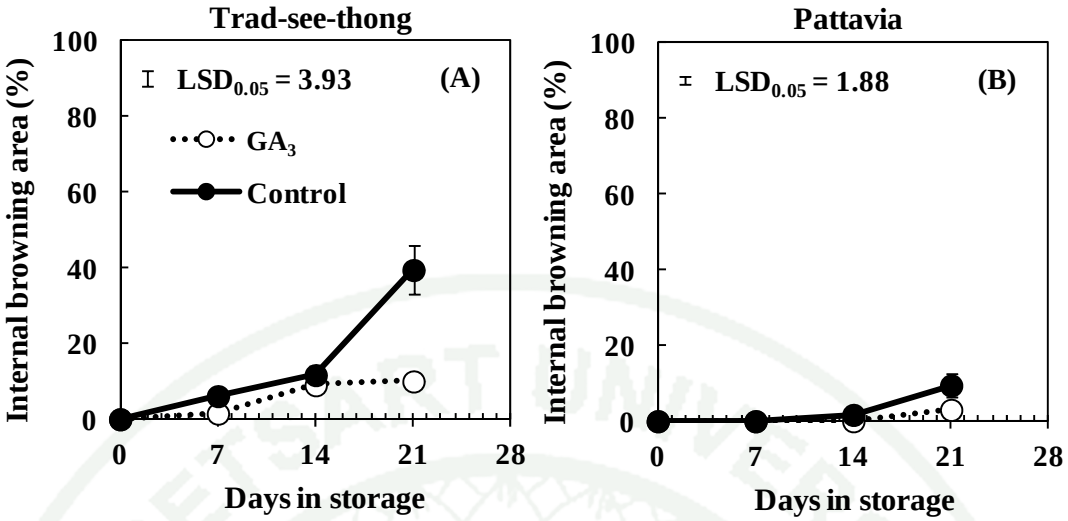

Figure 30 Internal browning area of Trad-see-thong (A) and Pattavia (B) pineapples treated with GA_3 and stored at 10 °C for 21 days. Data are mean ± SE of ten replications.

Additionally, the photographs of IB in both cultivar treated with GA_3 are shown in Figure 31.

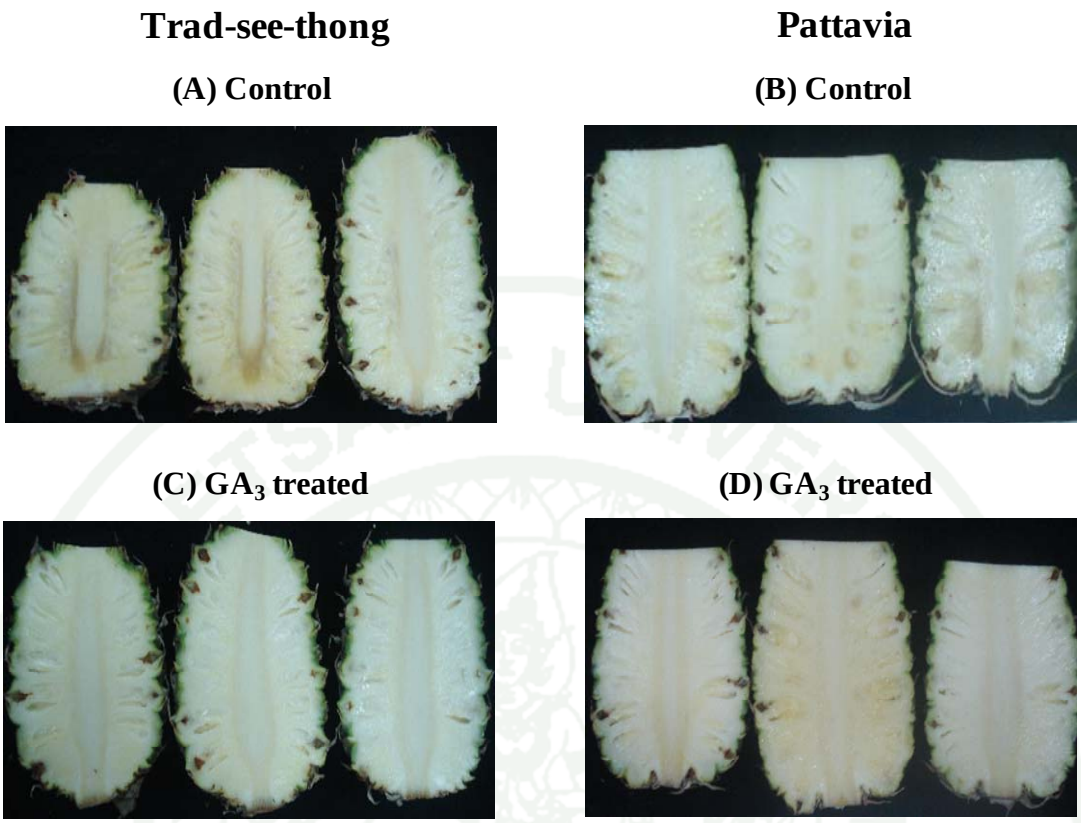

Figure 31 Internal browning of Trad-see-thong (A and C) and Pattavia (B and D) pineapples treated with GA_3 and stored at 10 °C for 21 days.

2) PPO activity

In Trad-see-thong, GA_3 application significantly reduced PPO activity during storage at 10 °C (Figure 32A). At day 21 during storage at 10 °C, PPO activity in fruit treated with GA_3 was 0.41 units/mg protein, while PPO activity in the control fruit was 0.67 units/mg protein. In Pattavia, GA_3 application was also reduced PPO activity during storage at 10 °C, but there was no significant difference ($P>0.05$) (Figure 32B). At day 21 during storage at 10 °C, PPO activity in fruit treated with GA_3 was 0.79 units/mg protein, while PPO activity in the control fruit was 0.84 units/mg protein.

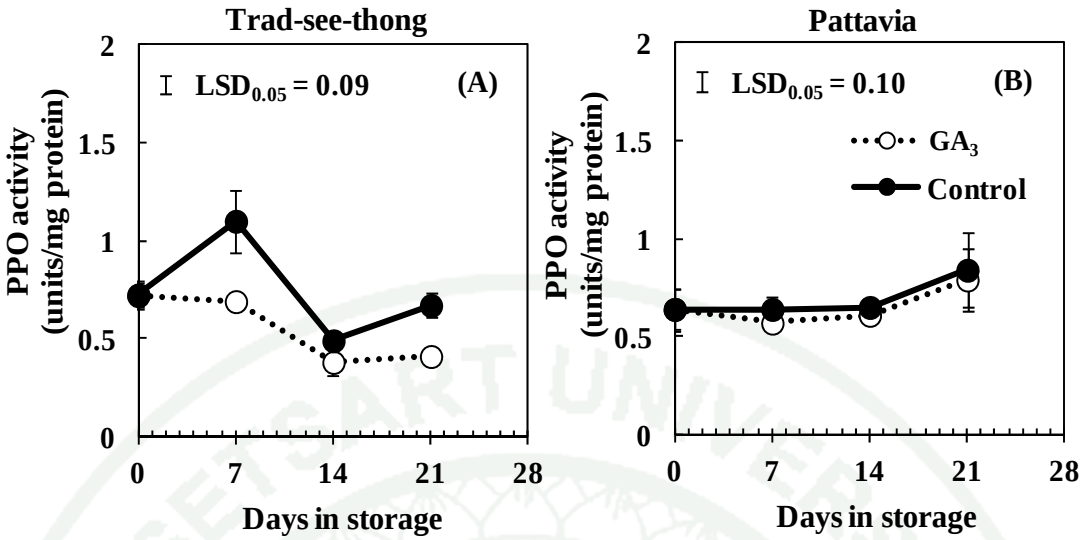

Figure 32 PPO activities of Trad-see-thong (A) and Pattavia (B) pineapples treated with GA_3 and stored at 10 °C for 21 days. Data are mean ± SE of three replications.

3) Endogenous ABA concentrations

In both cultivars, GA_3 application significantly reduced the endogenous ABA concentration (Figure 33A and 33B). In Trad-see-thong, endogenous ABA concentration in fruit treated with GA_3 was slightly reduced from 0.2 to 0.17 µmol/kg FW, while endogenous ABA concentration in the control fruit rapidly increased from 0.20 to 0.67 µmol/kg FW during storage at 10 °C (Figure 33A). In Pattavia, endogenous ABA concentration in fruit treated with GA_3 was slightly reduced from 0.09 to 0.03 µmol/kg FW while endogenous ABA concentration in the control fruit rapidly increased from 0.09 to 0.32 µmol/kg FW during storage at 10 °C (Figure 33B).

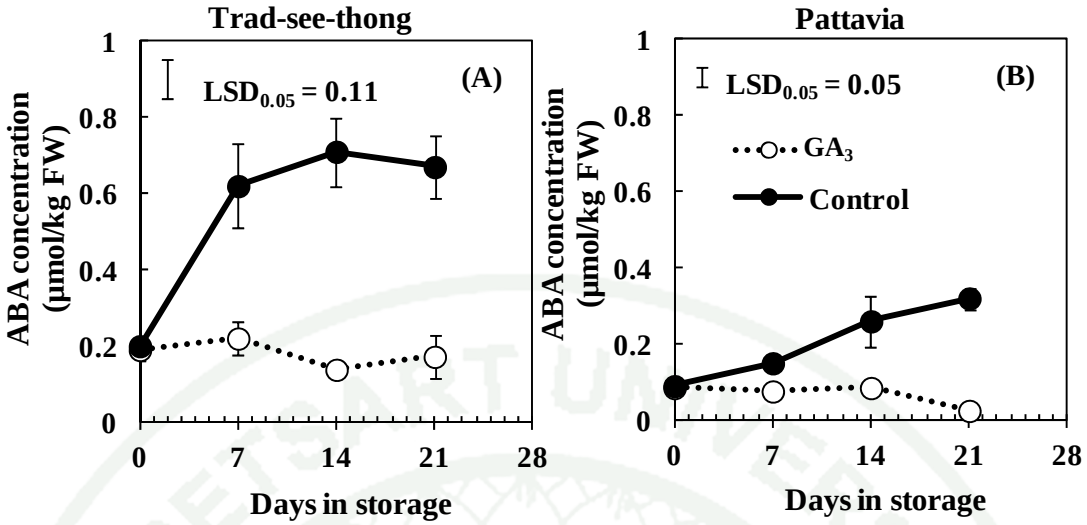

Figure 33 Endogenous ABA concentrations in Trad-see-thong (A) and Pattavia (B) pineapples treated with GA_3 and stored at 10 °C for 21 days. Data are mean ± SE of three replications.

Discussion

1. Role of calcium on IB of pineapple

In both Mauritius (Queen) and Kew (Smooth Cayenne) pineapples in Sri Lanka, the total calcium contents are higher in the pulp than those in the core, which are negatively correlated with the IB symptoms after storage at 10 °C for 17 days followed by 2 days at 28 °C (Hewajulige *et al*., 2003; Wilson Wijeratnam *et al*., 2006). Also in our experiment, total calcium contents were higher in the pulp than those in the core in both Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) pineapples. IB symptoms were lower in the pulp than in the core in Trad-see-thong (Queen) pineapple, similar to that earlier reported by Hewajulige *et al*. (2003). However, IB symptoms were higher in the pulp than in the core in Pattavia (Smooth Cayenne) pineapple. These results are in contrast with those reported by Hewajulige *et al*. (2003). Conversely, IB symptoms development in both Queen and Smooth Cayenne pineapples supported a previous report (Weerahewa and Adikaram, 2005a) that the patterns of IB symptoms in the Queen and Smooth Cayenne pineapples are different. The IB area in Mauritius (Queen) pineapple expanded along the core, while the IB area in Kew (Smooth Cayenne) pineapple mostly occurs in the pulp.

There were relationships between the total calcium content before storage and IB symptoms after storage at 10 °C for 21 days followed by 1 day at 25 °C. Both Queen and Smooth Cayenne pineapples harvested from Nakhon Pathom in the 2009 season contained very high calcium contents in the pulp and the core (more than1,500 mg/kg DW), showed no IB symptom at all (Figure 7). Also, in the 2011 season, Queen pineapple harvested in Nakhon Pathom, contained very high calcium contents in the pulp and the core (more than 1,100 mg/kg DW), they showed the lowest IB as compared to those from other growing locations (Figure 10).

The reason that high calcium contents were found in both pineapple groups from Nakhon Pathom is the soil pH. Soil pH affects the availability of soil nutrients and results in variable amounts of the various elements found in plants (Sims, 1986).

Nakhon Pathom soil series is neutral to slightly alkaline with pH 6.5-8.0 where calcium is readily available. In contrast, Chiangrai, Trad, Phetchaburi and Nakorn Ratchasima soil series are moderately acidic to slightly acidic with pH 5.5-6.5 and Rayong soil series are moderately acidic to neutral with pH 5.5-7.0. Availability of calcium is reduced in more acid soil (USDA Natural Resources Conservation Service, 1998; Land development department, 2014). In our experiment, pineapple from these acidic growing locations had predominantly lower than 500 mg/kg DW of calcium and developed some degree of IB, except Smooth Cayenne pineapple from Trad which had approximately 1,500 and 600 mg/kg DW of calcium in the pulp and the core, respectively, and showed no IB symptom in the pulp and slight IB in the core.

The above results support previous reports that pineapple with high calcium content have less IB symptoms (Herath *et al*., 2003; Hewajulige *et al*., 2003,), especially fruit with more than 500 mg/kg DW of calcium (Pitukwong, *et al*., 2013).

The reason that high calcium content reduces IB is probably caused by calcium maintaining the stability of the membranes (Poovaiah *et al*., 1988; Picchioni *et al*., 1995) and increases antioxidant and antioxidant enzyme activities. (Herath *et al*., 2003; Hernández *et al*., 2003; Singh *et al*., 2007; Mirdehghan and Ghotbi, 2014). Calcium ion (Ca^{2+}) stabilizes membranes and prevents solute leakage by interacting with the negative charges of the phosphate and the carboxylic groups of phospholipids and protein at the plasma membrane surface (Picchioni *et al*., 1995; Hirschi, 2004). An increase in the content of membrane-associated Ca^{2+} also reduces activities of lipid degradation enzymes, phospholipase D and lipoxygenase in cucumber fruit (*Cucumic sativus* L.) (Mao *et al*, 2007). Calcium also reduces IB by increasing ascorbic acid in Mauritius (Queen) pineapple (Herath *et al*., 2003). Pineapple containing high ascorbic acid has lower IB area than those with low ascorbic acid content (Pimpimol and Siriphanich, 1993; Herath *et al*., 2003).

When comparing within group and cultivar, Hewajulige *et al*. (2003) reported that there is negative correlation between calcium content and IB symptom in both Mauritius (Queen) ($r = 0.831$) and Kew (Smooth Cayenne)($r = 0.872$) pineapples.

Also in our experiment, there were negative correlation between calcium content and IB symptom in both Queen (r = -0.636) (Figure 8A) and Smooth Cayenne (r = -0.934) (Figure 8B) groups and also both Trad-see-thong (r = -0.966) (Figure 9A) and Pattavia (r = -0.935) (Figure 9B) pineapple. It should be noted, however that the data on IB and calcium are poorly distributed.

When comparing between group, Hewajulige *et al*. (2003) and Wilson Wijeratnam *et al*. (2006) reported that Queen pineapple developed more IB symptom and have less calcium content than Smooth Cayenne pineapple. In our experiment, Queen pineapple also developed more IB symptom than Smooth Cayenne pineapple, However, in this experiment, total calcium contents in Queen and Smooth Cayenne pineapples was not significantly different, expect in the pulp of Smooth Cayenne pineapple harvested from Trad that had a higher total calcium contents than Queen pineapple. Furthermore, total calcium content was higher in Queen pineapple than in Smooth Cayenne pineapple harvested in Nakhon Pathom. Additionally, both pineapple groups harvested from Chiangrai and Rayong had the same level of calcium contents in the pulp and the core but the IB development in the pulp and the core were totally different. Pineapple harvested from Rayong had moderate to severe IB symptoms, while fruit harvested from Chiangrai had only slight IB symptoms. These results are in contrast with those reported by Hewajulige *et al*. (2003) and Wilson Wijeratnam *et al*. (2006).

Pre-harvest calcium application reduced the IB of Mauritius (Queen) pineapple after storage at 10 °C for 17 days and followed by 2 day at 25 °C, by increasing the total calcium content in the pulp and the core (Wilson Wijeratanam *et al*., 2006). Also in our experiments, pre-harvest foliar, soil and combined foliar and soil calcium applications increased fruit calcium content, resulting in IB reduction. The fruit calcium contents were significantly different ($P \leq 0.05$) in experiment 1.2.1 and 1.2.2 but were not significantly different (P>0.05) in experiment 1.2.3. Foliar application increased calcium content in the pulp by as much as 45%, resulting in more than 35% IB reduction in the pulp after storage at 10 °C for 21 days followed by 1 day at 25 °C, but the results were not significantly different (P>0.05) in the core

(Figure 12). Soil application with 200 kg/ha CaO increased calcium content in pulp and the core by as much as 80% and 51%, respectively, resulting in more than 25% and 41% IB reduction in the pulp and the core after storage at 10 °C for 21 days followed by 1 day at 25 °C (Figure 13).

In experiment 1.2.3, combined foliar and soil application increased (not significant) the calcium contents in the pulp and the core by as much as 55% and 107%, respectively, but resulting in more than 50% and 70% IB reduction after storage at 10 °C for 21 days followed by 1 day at 25 °C (Figure 14). The foliar or soil application alone reduced IB in the core by 47% or 48%, respectively, but fruit calcium contents were not significantly different ($P>0.05$) from the control (Figure 14). It should be noted that the calcium content in pineapples in this experiment was rather high. Even in the control, the total content was more than 700 mg/Kg DW, about two times higher than that reported in 1.2.1, 1.2.2 and by Wilson Wijeratnam *et al*. (2006). More study is required, including an assessment of the cost-effectiveness of pre-harvest calcium application to reduce IB.

It should be noticed that after storage, the pulp color in calcium treated fruit remained pale yellow, while the control fruit became yellower (Figure 15). Similar finding were reported: pre-harvest calcium application, delays ripening and senescence in mango (*Mangifera indica* L.) (Singh *et al*., 1993) and strawberry (*Fragaria* × *ananassa* Duch.) (Singh *et al*., 2007), decreases the b value (yellowness) of surface fruit as compared with control fruit in mango (*Mangifera indica* L.) (Singh *et al*., 1993). It has also been reported that pre-harvest calcium application during fruit development can also delay senescence, resulting in the decrease in occurrence of translucency in pineapple, by increasing of calcium content in the pulp (Chen and Paull, 2001; Silva *et al*., 2006).

Postharvest calcium application by directly spraying on the fruit or by peduncle infiltration increase the total calcium content in the fruit and reduced the IB of Mauritius (Queen) pineapple (Nanayakkara *et al*., 2005) and Trad-see-thong pineapples (Youryon *et al*., 2013), respectively. In our experiment, postharvest stem

immersion, in calcium solution resulted in increased total calcium contents in both the pulp and the core from approximately 300 mg/kg DW in the control to more than 1,200 mg/Kg DW in the pulp and more than 3,000 mg/kg DW in the core, for the 4% $CaCl_2$ application. The IB area was reduced by as much as 4-fold (Figure 16). However, five repeated postharvest calcium experiments could not confirm the effect on IB reduction of pineapple (Figure 18 and Appendix Table 7 and 9-11).

Longer durations of low calcium concentration treatments, plus delaying the time after immersion until the time of exposure to low temperature by up to three days, were studied. This was to allow the calcium to increase its effect on membranes before exposure to cold stress. However, no reduction in IB could be achieved (Table 1 and Figure 20).

However, it should be noted that in experiment 1.3.1, the IB symptom in the control was rather mild. The affected area in the control fruit was only 10% in the pulp and 25% in the core. On the opposite, in experiment 1.3.2 and 1.3.3, the IB symptom in the control was rather high. The affected areas in the control fruit in experiments 1.3.2 and 1.3.3 were approximately 40-46% and 45-65%, respectively. Hence the inconsistencies in the calcium treatments may be due to other factor(s) existing in the fruit before treatments. In addition, high concentration of calcium in the immersion treatment caused a dark brown area in the fruit stem that extended into the core (Figure 17 and 19). This discoloration may limit its commercial application.

All of the result above indicates that calcium definitely has a role in the development of IB but other factors are also involved. Pre- or postharvest application with calcium could not guarantee reduction of IB. In addition, the higher susceptibility of Queen pineapple to IB as compare to Smooth Cayenne pineapple could not be explained simply by the fruit calcium content. Other factors that are reported to influence IB in pineapple are nutrients such as potassium (Nanayakkara *et al*., 2005; Soares *et al*., 2005), growing area condition (Smith and Glennie, 1987), weather conditions such as rainfall and temperature (Pitukwong, *et al*., 2013) and plant hormones such as GA_3 (Zhou and Tan 1997; Zhou *et al*., 2003b), ABA (Zhou

and Pan, 1997), methyl jasmonate (Nilpraprucк *et al*., 2009) and salicylic acid contents (Lu *et al*., 2011).

2. The role of endogenous GA and ABA in IB development in pineapple

The development of IB increased in Queen and Smooth Cayenne pineapple fruit after transferr to 25 °C following storage at 12-13 °C for 17-21 days (Stewart *et al*., 2001; Zhou *et al*., 2003a). Also in our experiment, the degree of IB of both Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) pineapples were higher in fruit transferred from 10 °C to 25 °C than in fruit stored at 10 °C, while IB was not found during storage at 25 °C (Figure 21). In both pineapple cultivars, the IB incidence was correlated with the increase in PPO activity (Figure 22). This result confirms that IB is associated with an increase in PPO activity, similar to earlier reports that PPO activity is induced in response to chilling (Stewart *et al*., 2001; Zhou *et al*, 2003a; Youryon *et al*., 2008; Lu *et al*., 2011).

In our experiment, PPO activity in Trad-see-thong pineapple was highest in fruit transferred from 10 °C to 25 °C, followed by fruit stored at 10 °C (Figure 19). The PPO amino acid sequences of Trad-see-thong pineapple and Pattavia pineapple are 99% identical (Chanapan *et al*., 2009). The *PINPPO1* and *PINPPO2* gene expressions and PPO activity slightly increases during storage at a low temperature but increases rapidly after the transferred from the low temperature to 25 °C in the Smooth Cayenne pineapple (Stewart *et al*., 2001). It has also been reported that *PPO* gene expression is up-regulated when apple fruit is transferred from 0 °C to the ambient temperature (Boss *et al*., 1995). The sequence homologue to element of the low-temperature-response (CCGAC) is found not in the *PINPPO1* promoter but in the *PINPPO2* promoter in the Smooth Cayenne pineapple (Zhou *et al*., 2003b). However, the expression level of the *PINPPO1* gene is higher than that in the *PINPPO2* gene during the subsequent period at 25 °C (Stewart *et al*., 2001).This result may explain why the IB increased in fruit transferred from 10 °C to 25 °C.

It has been shown that both the *PINPPO1* and *PINPPO2* genes of Smooth Cayenne pineapple also consist of complex sequence homologues to elements of the GA response complex consisting of the pyrimidine, GARE and Box I (Zhou *et al*., 2003b). In our experiment, endogenous GA concentration of both pineapple cultivars increased in fruit transferred from 10 °C to 25 °C, while endogenous GA concentration did not change in fruit stored at 10 °C and at 25 °C (Figure 23 and 24). Low temperature decreases the expression levels of GA biosynthesis genes, *GA20ox3* and *GA3ox1* in the development anthers of rice (*Oryza sativa*) (Sakata *et al*., 2014). In addition, low temperature increases both the relative expression levels of GA biosynthesis genes, *GA20ox1*, *GA20ox3*and *GA3ox2*, and GA deactivation genes, *GA2ox3* and *GA2ox6,* in *Arabidopsis thaliana* plant (Achard *et al*., 2008). After the transfer from 20°C to high temperature (29 °C), the expression of GA biosynthesis genes, *GA20ox1*and *GA3ox1*, are rapidly up-regulated, while the expression of GA deactivation gene, *GA2ox1*, decreases rapidly after the transfer, being 10 fold lower than control within 4 hours later in *Arabidopsis thaliana* seedling (Stavang *et al*., 2009). It has also been reported that cold tolerance is associated with the reduce endogenous GA_1 and GA_4 concentrations in bamboo (*Neosinocalamus affinis*) (Zhang *et al*., 2012). In our experiment, total endogenous GA ($GA_1 + GA_3 + GA_4$) concentration in fruit after transfer to 25 °C in Trad-see-thong pineapple was higher than in Pattavia pineapple. In addition, DELLA protein (negative regulator GA response) increases at low temperature (4 °C) and rapidly decreased after transferred to 23 °C in Arabidopsis seed (Lee *et al*., 2002). This result suggests that the increase in total endogenous GA ($GA_1 + GA_3 + GA_4$) concentration is associated with the increases in IB and PPO activity after the transfer to 25 °C.

GA_3 application increased the PPO activity in pineapples (Zhou and Tan, 1997) and induces PPO gene expression and IB development in Smooth Cayenne pineapples in the absence of chilling (Zhou *et al*., 2003b). Also in our experiment during storage at 25 °C, GA_3 application increased both IB (Figure 26 and 27) and PPO activity (Figure 28) of the both Trad-see-thong (Queen) and Pattavia (Smooth Cayenne) pineapples stored at 25 °C. In addition, the degree of IB with GA_3 application in Trad-see-thong (Queen) pineapple was higher than in Pattavia (Smooth Cayenne) pineapple.

In the presence of low bioactive GA concentration, DELLA protein is relatively stable (Achard and Genschik, 2009). In contrast, in the presence of high bioactive GA concentration, the GA receptor is bound by GA, thus stimulated ubiquitination and destruction of DELLA protein to promote binding with a specific SCF (F-box, Skp1 and Cullin) E3 ubiquitin-ligase, by 26S proteasome (Achard and Genschik, 2009). GA_3 application rapidly decreases the level of DELLA protein in barley aleurone cells (*Hordeum vulgare*) (Gubler *et al*., 2002; Ishibashi *et al*., 2012) and cotton plant (*Gossypium hirsutum* L.) (Aleman *et al*., 2008), which results in the induction of *GAMYB* mRNA in barley aleurone cells (*Hordeum vulgare*) (Gubler *et al*., 2002). The increase in the expression of *GAMYB* gene activateds several GA-induce genes such as α-amylase gene in barley aleurone cells in the presence of GA (*Hordeum vulgare*) (Gubler *et al*., 2002; Woodger *et al*., 2003), as well as in transgenic barley aleurone cells (*Hordeum vulgare*) in the absence of exogenous GA_3 (Gubler *et al*., 1995). It has also been shown that GAMYB transcription factor binds specifically to the GARE (TAACAAA) of α-amylase gene promoter to induce α-amylase gene expression in barley aleurone cells (*Hordeum vulgare*) (Gubler *et al*., 1995). Moreover, both the *PINPPO1* and *PINPPO2* genes, which are not found only GARE but also GAMYB binding site, were GA-responsive in Smooth Cayenne pineapple (Zhou *et al*., 2003b). Therefore, it is possible that the increases in IB and induction of PPO activity in pineapples after transfer to 25 °C may be related to the increases in endogenous GA concentrations.

At 10 °C, GA_3 application reduces IB in Smooth Cayenne pineapple (O'Hare *et al*., 2001). Also in this experiment at 10°C, GA_3 application reduced both IB (Figure 30 and 31) and PPO activity (Figure 32) in both pineapple cultivars stored at 10 °C. These results are in contrast with both our work (at 25 °C) and those earlier reported (at 25 °C) by Zhou and Tan (1997) and Zhou *et al*., (2003b). In addition, low temperature decreases the active GA level by increasing the transcript levels of GA deactivation genes, *GA2ox3* and *GA2ox6*, increases GA2ox enzyme level and increases the accumulation of DELLA protein in *Arabidopsis thaliana* plant (Achard *et al*., 2008). Low temperature also increases the accumulation of DELLA protein (by increasing their stability) in anther of rice (*Oryza sativa*) (Sakata *et al*., 2014).These

results indicates that the induction of IB and PPO activity during storage at 10 °C in pineapples may not be related to the change in endogenous GA concentration, as it was not the same as the result at 25 °C. Therefore, it is possible that the increases in IB and induction of PPO activity in pineapples at 10 °C may not involve GA.

ABA concentration increases under low temperature condition in zucchini squash (*Cucurbita pepo* L.) (Wang and Buta, 1994). In our experiment, in both pineapple cultivars, endogenous ABA concentrations increased during storage at 10 °C and decreased after the fruits were transferred from 10 °C to 25 °C (Figure 25). ABA catabolism is largely categorized into two types of reaction, hydroxylation by ABA 8′-hydorxylase and conjugation by ABA glucosidase (Kushiro *et al*., 2004; Nambara and Marion-Poll, 2005; Burla *et al*., 2013). An increase in endogenous ABA concentrations in chilled fruit and a decrease in fruit transferred to 20 °C has also been reported in the tomato (Ludford and Hillman, 1990). It has been shown that ABA plays a role in stress signal transduction, which is associated with the defense system in plants and which induces the secondary metabolite production against stress (Wang *et al*., 2008; Kondo *et al*., 2011). Cold-sensitive rice (*Oryza sativa*) also accumulates higher ABA than cold-tolerant rice under cold conditions (Oliver *et al*., 2007). In our experiment, endogenous ABA concentration was also higher in Trad-see-thong pineapple than in Pattavia pineapple during storage at 10 °C. This suggests that the increases in the endogenous ABA concentrations may be a low-temperature-tolerance mechanism to reduce IB and PPO activity in pineapples. Exogenous ABA application delays IB and PPO activity in Smooth Cayenne pineapple (Zhou and Pan, 1997). In addition, ABA inhibits the GA biosynthesis genes, *GA20ox1*and *GA3ox1*, in *Arabidopsis thaliana* seed (Toh *et al*., 2008) and sunflower (*Helianthus annuus*) hypocotyls (Kurepin *et al*., 2011).

In the experiment at 25 °C, GA_3 application dramatically increased the endogenous ABA concentration in Trad-see-thong pineapple at day 14, then it rapidly decreased while IB continued to increase after day 14. In Pattavia pineapple, GA_3 application significantly increased the endogenous ABA concentration from day 7 until day 14 (Figure 29), but to a much smaller degree than that found in Trad-see-

thong pineapple. A reduction of IB and the delay of the increase in PPO activity were observed in ABA-treated pineapple after storage at chilling temperature (Zhou and Pan, 1997). ABA also inhibits the expression of transcription activator of GA-regulator, *GAMYB*, gene, in barley aleurone cells (*Hordeum vulgare*) (Gubler *et al*., 2002). In the experiment at 10 °C, GA_3 application inhibited the increase in endogenous ABA concentration and delayed the increase of IB (Figure 33). This result suggests that ABA has a role in IB development, perhaps as a plant response against environmental stress.

Low temperature or exogenous ABA application also induces the expression of C-repeat binding factor (*CBF1*, *CBF2* and *CBF3*) genes in *Arabidopsis thaliana* (Medina *et al*., 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Knight *et al*., 2004), freezing-tolerant wild grapes (*Vitis riparia* Michx.) and freezing-sensitive cultivated grapes (*Vitis vinifera* L.) (Xiao *et al*., 2006). *CBF1*, *CBF2* and *CBF3* genes encode CBFs transcription factor that bind to the LTRE (CCGAC) in the promoter of cold-inducible genes in *Arabidopsis thaliana* (Gilmour *et al*., 1998), *Brassica napus*, wheat (*Triticum aestivum* L.) rye (*Secale cereale* L.) and tomato (*Lycopersicon esculentum*) (Shinozaki and Yamaguchi-Shinozaki, 2000; Jaglo *et al*., 2001). In Smooth Cayenne pineapple, *PINPPO2* genes promoter LTRE (CCGAC) is also found (Zhou *et al*., 2003b). This result indicates that the induction of IB and PPO activity during storage at 10 °C in pineapples, by increasing the expression of *PINPPO2* gene, may be through an increase in *CBF* genes expression in the ABA-independent and ABA-dependent manners. In the experiment at 10 °C, GA_3 application decreased IB and PPO activity and inhibited the increases in endogenous ABA concentrations of both pineapple cultivars during storage at 10 °C (Figure 30). Low temperature or CBF1 protein also induces the accumulation of DELLA protein by stimulating the expression of GA deactivation enzyme, GA2ox, and decreases endogenous active GA concentration in *Arabidopsis thaliana* plant (Achard *et al*., 2008). This result suggest that the reduction of IB and PPO activity during storage at 10 °C in GA-treated pineapple may be related to the decreased *CBF* genes expression by inhibiting the increase in endogenous ABA concentration. Moreover, Zentella *et al*. (2007) and Seo

et al. (2009) suggested that GA and ABA reduce each other's synthesis in *Arabidopsis* seedling and seed, respectively.

Although the data on GA and ABA concentration in pineapple under cold storage and after exogenous GA treatment indicates that they have roles in IB development, their functions are not yet clear. The interpretation models for the IB development in pineapple and GA and ABA involvement under four situations are shown in Appendix Figure 1-4.

Anyhow, more works are required to elucidate their roles. The use of GA action inhibitor, ABA, or ABA action inhibitors on pineapple before storage at low temperature could provide some explanations

On the association between calcium, GA and ABA, Kuo *et al*. (1996) reported that calcium application alone delays cell damage and reduces the expression of GA-induced α-amylase gene in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) aleurone layers during seed germination. Furthermore, the GA_{4+7} application during development of tomato fruit (*Solanum lycopersicum*) reduces water-soluble apoplastic calcium concentration, resulting in increasing cytosol calcium concentration and fruit cell membrane leakage or cell damage (De Freitas *et al*., 2012). Conversely, ABA application during development of tomato fruit increases fruit total and water-soluble apoplastic calcium concentrations and reduces fruit cell membrane damage (De Freitas *et al*., 2011). Further works are required to show the associate between calcium and GA and ABA in the development of IB in pineapple.

CONCLUSIONS

The study on the role of calcium, gibberellins and abscisic acid on internal browning of pineapple can be concluded in the following:

1. Total calcium contents before storage in Queen and Smooth Cayenne pineapples, harvested from various growing areas in Thailand, had a negative correlation with IB development after storage at 10 °C for 21 days. Total calcium was lower in Queen pineapple than in Smooth Cayenne pineapple from Trad. However, total calcium content was higher in Queen pineapple than in Smooth Cayenne pineapple from Nakhon Pathom province. Pre-harvest calcium application by foliar or soil application alone and foliar together with soil application increased the fruit calcium contents and reduced IB of Trad-see-thong pineapple after storage at 10 °C for 21 days. However, the fruit calcium content was not significantly different in some experiments.

2. In Trad-see-thong pineapple, postharvest $CaCl_2$ application by immersion only the stem in 2% and 4% $CaCl_2$ solutions at 25 °C for 18 hours reduced IB by 2 and 4-fold after storage at 10 °C for 21 days. However, five repeated experiments could not show the effectiveness of calcium to reduce IB. In addition, immersion only the stem in 0.5-1.5 % $CaCl_2$ solutions at 25 °C for 24-72 hours had no effect on IB after storage at 10 °C for 21 days.

3. Calcium content is concluded to be only one of many factors influencing IB in pineapple.

4. The increases in IB and PPO activity were found in pineapple at low temperatures. IB and PPO activity rapidly increased in the fruit of both Trad-see-thong and Pattavia pinepples after transferred from 10 °C to 25 °C. Trad-see-thong pineapple had higher IB and PPO activity than those in Pattavia pineapple.

5. In both Trad-see-thong and Pattavia pineapples, total endogenous GA ($GA_1 + GA_3 + GA_4$) concentrations were not associated with increases in IB and PPO activity in fruit stored at 10 °C. However, increases in total endogenous GA concentrations were associated with increases in IB and PPO activity in fruit transferred from 10 °C to 25 °C.

6. In both Trad-see-thong and Pattavia pineapples, GA_3 application by fruit dipping in GA_3 solution for 5 minutes significantly induced both IB and PPO activity during storage at 25 °C. In contrast at 10 °C, GA_3 application significantly reduced IB and PPO activity.

7. In both Trad-see-thong and Pattavia pineapples, endogenous ABA concentration increased in fruit stored at 10 °C and immediately decreased after transferred to 25 °C. The changes in endogenous ABA concentrations were ascribed to stress response.

8. GA_3 application by fruit dipping in GA_3 solution for 5 min significantly increased the endogenous ABA concentration in Trad-see-thong pineapple, stored at 25 °C. In contrast at 10 °C, GA_3 application significantly inhibited the increase in endogenous ABA concentration. Endogenous ABA concentration after GA_3 application in Trad-see-thong pineapple was higher than in Pattavia pineapple at both 10 °C and 25 °C.

9. In conclusion, GA and ABA are associated with the development of IB and PPO activity in pineapple but their actions are unclear.

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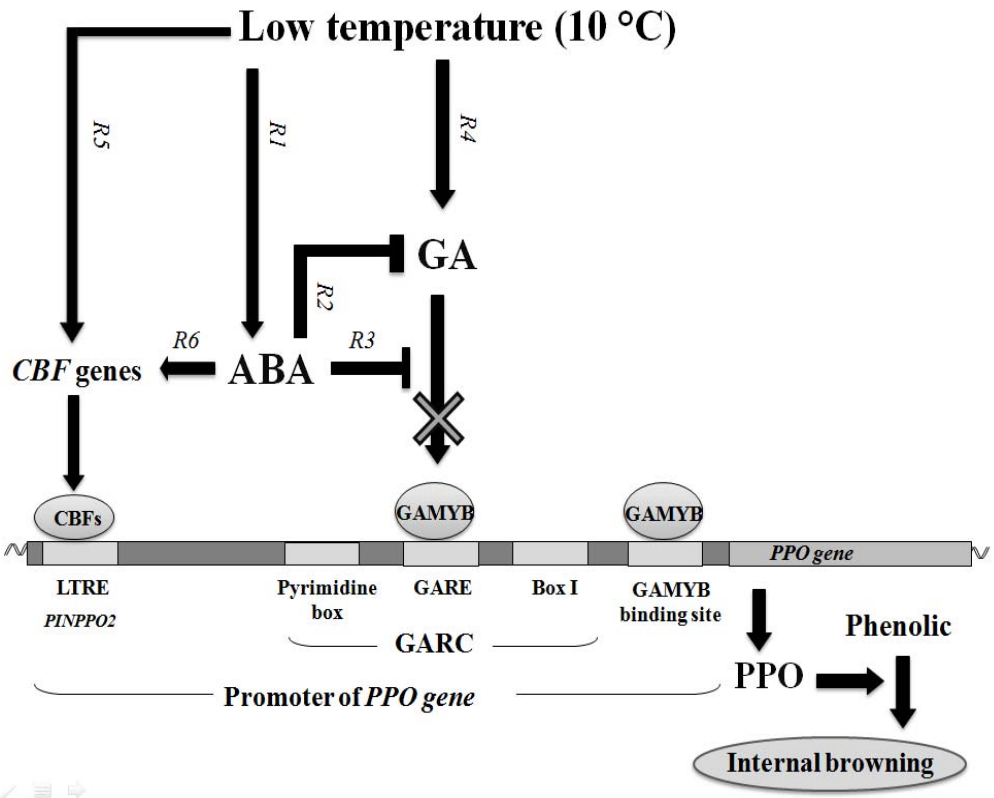
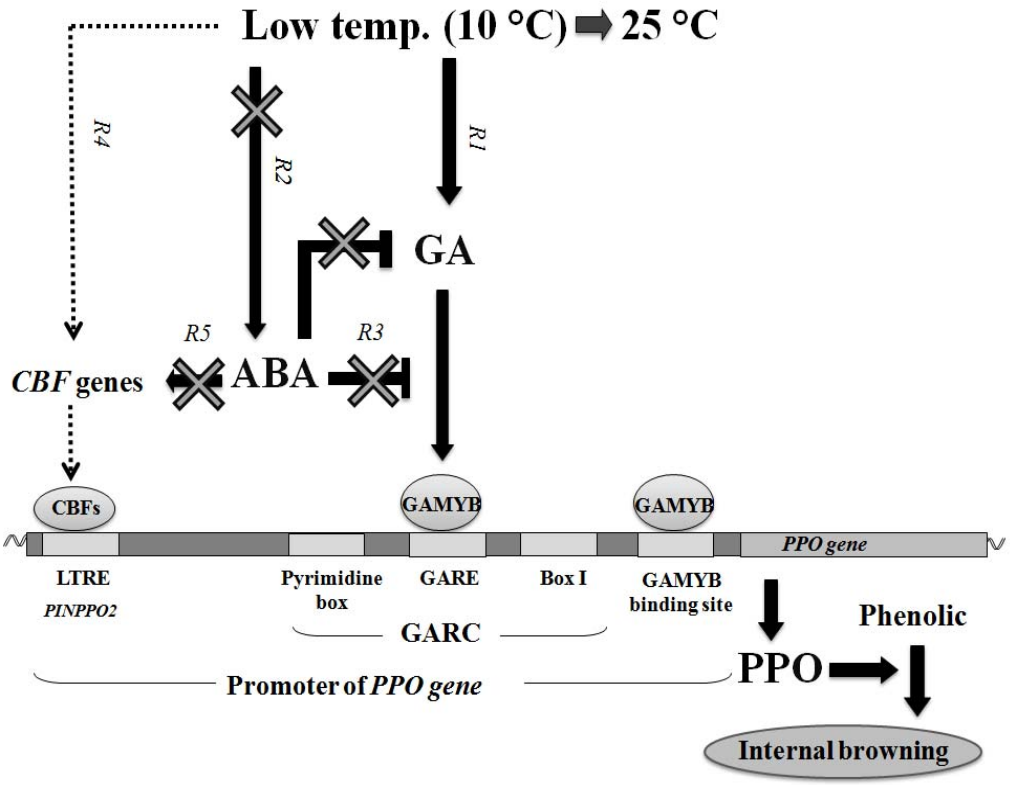
APPENDIX



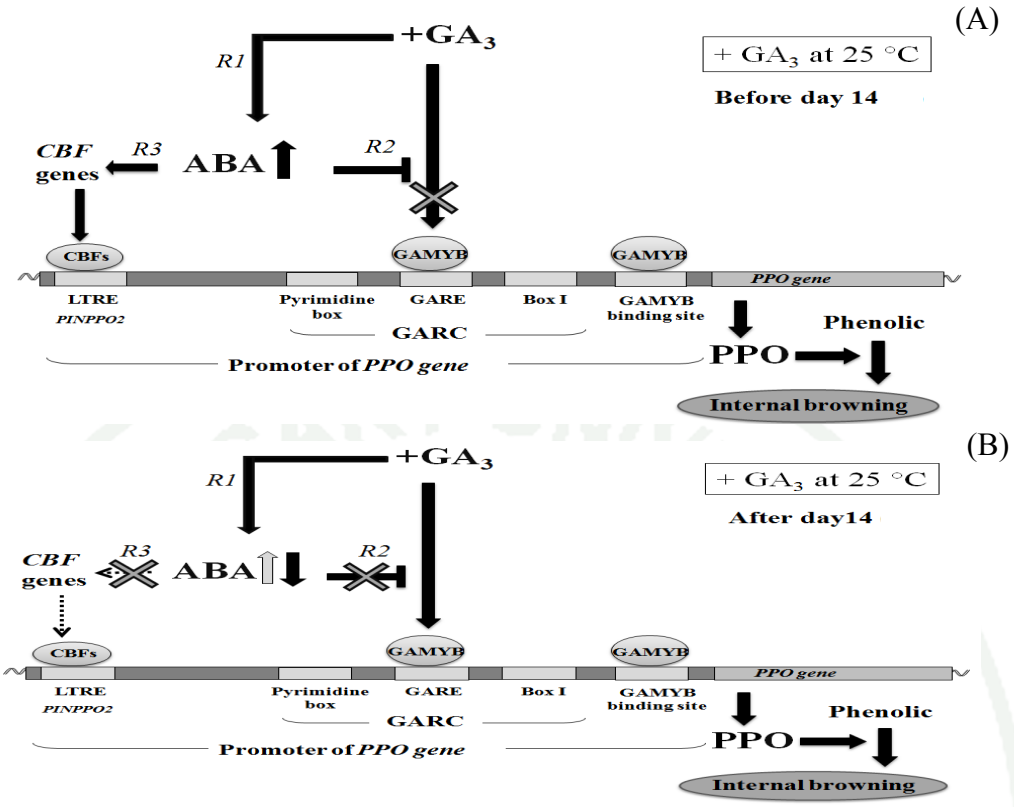
Appendix Figure 1 The interpretation model of GA and ABA signaling pathways which involved with IB development of pineapple during storage at 10 °C; low temperature induced the production of ABA (*R1*). An increase of endogenous ABA concentration may inhibit the increase of endogenous GA concentration (*R2*) together with the action of GA (*R3*) on IB development. This indicates that the induction of PPO activity and IB at low temperature are not associated with GA. The induction of PPO activity and IB may be through an increase in *CBF* genes expression under low temperature stress in ABA-independent (*R5*) and ABA-dependent manners (*R6*).

Note: *R1* = Table 25; Wang and Buta, 1994, *R2* = Toh *et al*., 2008; Kurepin *et al*., 2011, *R3* = Gubler *et al*., 2002, *R4* = Table 23 and 24; Achard *et al*., 2008, *R5* = Shinozaki and Yamaguchi-Shinozaki, 2000; Skinner *et al*., 2005, *R6* = Knight *et al*., 2004; Xiao *et al*., 2006



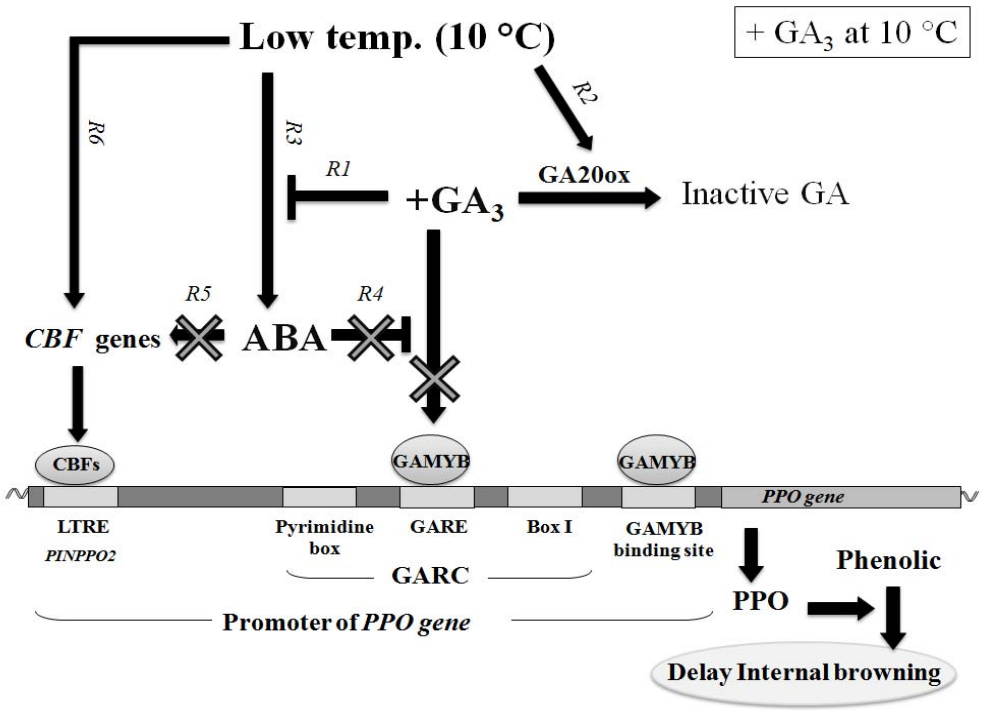
Appendix Figure 2 The interpretation model of GA and ABA signaling pathways which involved with IB development of pineapple after transfer from 10 °C to 25 °C; endogenous GA concentration increased (*R1*) and endogenous ABA concentration decreased (*R2*). The decrease of endogenous ABA concentration results in no inhibition of the increase of endogenous GA concentration and the action of GA on IB development. This indicates that the induction of PPO activity and IB after transfer from 10 °C to 25 °C associated with the increase in endogenous GA concentration and may also be though an earlier increase in *CBF* genes expression in ABA-independent manner (*R4*).

Note: *R1* = Table 23 and 24; Stavang *et al*., 2009, *R2* = Table 25; Ludford and Hillman, 1990, *R3* = Gubler *et al*., 2002, *R4* = Shinozaki and Yamaguchi-Shinozaki, 2000; Skinner *et al*., 2005, *R5* = Knight *et al*., 2004; Xiao *et al*., 2006, ⋯► = during storage at 10 °C



Appendix Figure 3 The interpretation model of GA and ABA signaling pathways which involved with IB development of pineapple during storage at 25 °C after GA_3 application; endogenous ABA concentration increased as a result of GA induction until day 14 and decreased after day 14 during storage (*R1*). Form day 0 to day 14 (A), the increase of endogenous ABA concentration may inhibited the action of GA (*R2*) on IB development. This indicates that the induction of IB and PPO activity before day 14 during storage at 25 °C may not be associated with GA but may be through an increase in *CBF* genes expression in ABA-dependent manner (*R3*). After day 14 (B), endogenous ABA decreased and no longer inhibited the action of GA on IB development. This indicates that the induction of PPO activity and IB after day 14 during storage at 25 °C is associated with the increase in endogenous GA concentration.

Note: *R1* = Table 29, *R2* = Gubler *et al*., 2002, *R3* = Knight *et al*., 2004; Xiao *et al*., 2006.
⋯► = during from day 0 to day 14



Appendix Figure 4 The interpretation model of GA and ABA signaling pathways which involved with IB development of pineapple during storage at 10 °C after GA_3 application; exogenous GA_3 inhibited the increase of endogenous ABA concentration (*R1*). Less endogenous ABA concentration could not inhibit the action of GA on IB development. However, GA_3 application at 10 °C delayed the induction of PPO activity and IB. The possibility is that low temperature may deactivate active GA to inactive GA by increasing the induction of GA deactivation enzyme, GA2ox (*R2*). The later PPO activity and IB induction may be through an increase in *CBF* genes expression under low temperature stress in ABA-independent manner (*R6*).

Note: *R1* = Table 33; Zentella *et al*., 2007; Seo *et al*., 2009, *R2* = Achard *et al*., 2008, *R3* = Table 25; Wang and Buta, 1994, *R4* = Gubler *et al*., 2002, *R5* = Knight *et al*., 2004; Xiao *et al*., 2006, *R6* = Shinozaki and Yamaguchi-Shinozaki, 2000; Skinner *et al*., 2005

Appendix Table 1 Total calcium contents (mg/kg DW) before storage and internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Queen and Smooth Cayenne pineapples harvested from four different locations.

Growing locations	Tissues	Total calcium content[1] (mg/kg DW)	Internal browning area[1] (%)
Phulea from Chiangrai	pulp	581.33d	3.30e
	core	302.21d	11.11de
Trad-see-thong from Rayong	pulp	392.48d	25.55cd
	core	236.50d	78.89a
Trad-see-thong from Trad	pulp	651.41d	50.00b
	core	206.83d	80.22e
Trad-see-thong from Nakhon pathom	pulp	4356.34a	0.00e
	core	2469.28b	0.00e
Nanglea from Chiangrai	pulp	525.14d	6.66e
	core	426.63d	3.33e
Pattavia from Rayong	pulp	305.04d	33.39c
	core	93.91d	11.11de
Pattavia from Trad	pulp	1539.34c	0.00e
	core	656.80d	4.44e
Pattavia from Nakhon Pathom	pulp	2426.24b	0.00e
	core	1565.09c	0.00e
CV (%)		35.46	77.09
F-test		**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

Appendix Table 2 Total calcium contents (mg/kg DW) before storage and internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples harvested from four different locations.

Growing location	Tissue	Total calcium content[1] (mg/kg DW)	Internal browning area[1] (%)
Trad-see-thong from Phetchaburi 1	pulp	403.00c	50.00ab
	core	297.58c	58.00a
Trad-see-thong from Phetchaburi 2	pulp	494.81c	24.00cd
	core	452.77c	36.00bc
Trad-see-thong from Nakhon Ratchasima	pulp	407.99c	40.00bc
	core	322.74c	48.50ab
Trad-see-thong from Nakhon pathom	pulp	2301.35c	18.00e
	core	1190.03b	27.50cd
CV (%)		31.63	48.88
F-test		**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

Appendix Table 3 Total calcium contents (mg/kg DW) at harvest and internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples after foliar calcium application.

Treatment	Tissue	Total calcium content[1] (mg/kg DW)	Internal browning area[1] (%)
Control	pulp	239.13b	31.25b
	core	198.60b	47.08a
Foliar application	pulp	357.11a	20.83c
	core	175.42b	44.17a
CV (%)		21.00	23.64
F-test		**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

Appendix Table 4 Total calcium contents (mg/kg DW) at harvest and internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples after soil calcium applications.

Treatment	Tissue	Total calcium content[1] (mg/kg DW)	Internal browning area[1] (%)
Control	pulp	407.99b	40.00ab
	core	322.74b	48.50a
100 kg/hectare Calcium oxide	pulp	745.49a	31.67abc
	core	351.91b	37.00abc
200 kg/hectare Calcium oxide	pulp	917.58a	30.38bc
	core	488.46b	28.08c
CV (%)		24.54	50.86
F-test		**	*

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

* = significantly different at $0.01 < P \leq 0.05$

** = significantly different at $P \leq 0.01$

Appendix Table 5 Total calcium contents (mg/kg DW) at harvest and internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples after different pre-harvest calcium applications.

Treatment	Tissue	Total calcium content[1] (mg/kg DW)	Internal browning area[1] (%)
Control	pulp	1351.80abc	25.68a
	core	707.08c	26.77a
Foliar application	pulp	1494.79ab	22.18ab
	core	876.57bc	14.23bc
Soil application	pulp	1664.92ab	19.47ab
	core	1053.75bc	14.07bc
Foliar and soil application	pulp	2105.226a	11.17bc
	core	1468.75bc	7.73c
CV (%)		35.66	44.53
F-test		*	*

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

* = significantly different at $P \leq 0.05$

Appendix Table 6 Total calcium contents (mg/kg DW) without and with immersion and internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0, 1, 2 and 4 % $CaCl_2$ solutions for 18 hours (First experiment).

Treatment	Tissue	Total calcium content[1] (mg/kg DW)	Internal browning area[1] (%)
without immersion	pulp	572.18cde	-
	core	359.92de	-
0% $CaCl_2$	pulp	262.73e	11.11bc
	core	306.87de	25.56a
1% $CaCl_2$	pulp	789.75cde	7.22cd
	core	1078.65cd	17.22b
2% $CaCl_2$	pulp	963.41cde	3.89cd
	core	1893.73b	11.11bc
4% $CaCl_2$	Pulp	1209.29bc	2.22d
	core	3237.49a	6.11cd
CV (%)		38.73	75.31
F-test		**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

Appendix Table 7 Internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0, 1, 2 and 4 % $CaCl_2$ solutions for 18 hours (Second experiment).

Treatment	Tissue	Internal browning area[1] (%)
without immersion	pulp	-
	core	-
0% $CaCl_2$	pulp	79.50ab
	core	88.50a
1% $CaCl_2$	pulp	73.00b
	core	73.50b
2% $CaCl_2$	pulp	79.00ab
	core	81.10ab
4% $CaCl_2$	Pulp	72.00b
	core	86.00ab
CV (%)		17.70
F-test		*

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

* = significantly different at $P \leq 0.05$

Appendix Table 8 Internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0 and 2 % $CaCl_2$ solutions for 18 hours (First experiment).

Treatment	Tissue	Internal browning area[1] (%)
0% $CaCl_2$	pulp	40.00
	core	46.50
2% $CaCl_2$	pulp	55.00
	core	45.50
CV (%)		62.79
F-test		ns

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

Appendix Table 9 Internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0 and 2 % $CaCl_2$ solutions for 18 hours (Second experiment).

Treatment	Tissue	Internal browning area[1] (%)
0% $CaCl_2$	pulp	44.00
	core	39.50
2% $CaCl_2$	pulp	46.50
	core	37.50
CV (%)		49.68
F-test		ns

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

Appendix Table 10 Internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0 and 2 % $CaCl_2$ solutions for 18 hours (Third experiment).

Treatment	Tissue	Internal browning area[1] (%)
0% $CaCl_2$	pulp	48.50
	core	46.00
2% $CaCl_2$	pulp	43.00
	core	43.50
CV (%)		55.07
F-test		ns

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

Appendix Table 11 Internal browning area (%) after storage at 10 °C for 21 days followed by 1 day at 25 °C in the pulp and the core of Trad-see-thong pineapples; stem submersion in 0 and 2 % $CaCl_2$ solutions for 18 hours (Forth experiment).

Treatment	Tissue	Internal browning area[1] (%)
0% $CaCl_2$	pulp	49.50
	core	55.50
2% $CaCl_2$	pulp	54.00
	core	60.00
CV (%)		43.99
F-test		ns

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

Appendix Table 12 Internal browning area (%) of Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.00	6.11ab	11.67b	39.44ab
10 °C + 1 d 25 °C	0.00	11.63a	26.11a	50.00a
25 °C	0.00	0.00b	0.00c	0.00b
CV (%)	0	116.17	45.53	52.70
F-test	ns	**	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

****** = significantly different at $P \leq 0.01$

Appendix Table 13 Internal browning area (%) of Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.00	0.00	1.67ab	9.44b
10 °C + 1 d 25 °C	0.00	0.00	4.44a	22.22a
25 °C	0.00	0.00	0.00c	0.00c
CV (%)	0	0	201.61	63.46
F-test	ns	ns	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

** = significantly different at $P \leq 0.01$

Appendix Table 14 PPO activities (units/mg protein) of Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.48	0.30b	1.00a	1.23b
10 °C + 1 d 25 °C	-	0.66a	0.19a	1.59a
25 °C	0.48	0.44ab	0.50b	0.78c
CV (%)	28.54	30.83	18.32	18.63
F-test	ns	**	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

** = significantly different at $P \leq 0.01$

Appendix Table 15 PPO activities (units/mg protein) of Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.42	0.38	0.84b	0.74b
10 °C + 1 d 25 °C	-	0.48	0.84b	0.83b
25 °C	0.42	0.40	0.40a	0.43a
CV (%)	17.11	13.01	12.53	8.98
F-test	ns	ns	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

** = significantly different at $P \leq 0.01$

Appendix Table 16 Endogenous GA_1 concentrations (µmol/kg FW) in Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.10	0.23a	0.79a	0.25b
10 °C + 1 d 25 °C	-	0.08b	0.39b	1.80a
25 °C	0.10	0.05b	0.08c	0.05b
CV (%)	9.74	36.88	24.37	26.32
F-test	ns	**	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

** = significantly different at $P \leq 0.01$

Appendix Table 17 Endogenous GA_1 concentrations (µmol/kg FW) in Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.05	0.06b	0.30	0.11b
10 °C + 1 d 25 °C	-	0.25a	0.32	1.65a
25 °C	0.05	0.04b	0.23	0.12b
CV (%)	19.54	14.79	94.05	44.19
F-test	ns	*	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

***** = significantly different at $0.01 < P \leq 0.05$

****** = significantly different at $P \leq 0.01$

Appendix Table 18 Endogenous GA_3 concentrations (µmol/kg FW) in Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	1.19	1.34	1.33b	1.02b
10 °C + 1 d 25 °C	-	1.29	3.48a	3.41a
25 °C	1.19	1.26	0.57b	0.63b
CV (%)	62.42	40.57	22.63	35.29
F-test	ns	ns	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

****** = significantly different at $P \leq 0.01$

Appendix Table 19 Endogenous GA_3 concentrations (µmol/kg FW) in Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	1.08	2.07a	1.40a	0.60
10 °C + 1 d 25 °C	-	2.07a	1.20ab	1.22
25 °C	1.08	1.23b	0.27b	1.71
CV (%)	44.66	11.43	62.18	61.78
F-test	ns	**	ns	ns

[1] Mean±SE value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

****** = significantly different at $P \leq 0.01$

Appendix Table 20 Endogenous GA_4 concentrations (µmol/kg FW) in Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.82	0.67b	0.49c	0.75ab
10 °C + 1 d 25 °C	-	2.18a	1.75a	1.06a
25 °C	0.82	0.77b	0.66b	0.57c
CV (%)	42.61	10.12	7.31	20.76
F-test	ns	**	**	*

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

***** = significantly different at $0.01 < P \leq 0.05$

****** = significantly different at $P \leq 0.01$

Appendix Table 21 Endogenous GA_4 concentrations (µmol/kg FW) in Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.35	0.50b	0.49	0.55
10 °C + 1 d 25 °C	-	1.30a	0.55	0.50
25 °C	0.35	0.53b	0.52	0.68
CV (%)	71.04	40.05	20.02	39.64
F-test	Ns	*	ns	ns

[1] Mean±SE value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

***** = significantly different at $P \leq 0.05$

Appendix Table 22 Total GA (endogenous $GA_1 + GA_3 + GA_4$) concentrations (mg/kg FW) in Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.72	0.77b	0.78b	0.69b
10 °C + 1 d 25 °C	-	1.20a	1.92a	2.16a
25 °C	0.72	0.71b	0.45c	0.43b
CV (%)	19.25	15.91	7.97	18.56
F-test	ns	*	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

***** = significantly different at $0.01 < P \leq 0.05$

****** = significantly different at $P \leq 0.01$

Appendix Table 23 Total GA (endogenous $GA_1 + GA_3 + GA_4$) concentrations (mg/kg FW) in Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.72	1.29a	0.80b	0.75b
10 °C + 1 d 25 °C	-	1.55a	1.00a	1.61a
25 °C	0.72	0.75b	0.42c	0.98b
CV (%)	21.01	23.93	9.56	17.04
F-test	ns	**	**	**

[1] Mean±SE value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

** = significantly different at $P \leq 0.01$

Appendix Table 24 Endogenous ABA concentrations (µmol/kg FW) in Trad-see-thong pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.20	0.62a	0.71a	0.67a
10 °C + 1 d 25 °C	-	0.14b	0.17b	0.48b
25 °C	0.20	0.06b	0.27b	0.13c
CV (%)	15.70	43.03	26.14	20.92
F-test	ns	**	**	**

[1] Mean value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

** = significantly different at $P \leq 0.01$

Appendix Table 25 Endogenous ABA concentrations (µmol/kg FW) in Pattavia pineapples during storage at 10 °C, 10 °C and transferred to 25 °C for 1 day (10 °C + 1 d 25 °C) and 25 °C.

Treatment	Days in storage[1]			
	0	7	14	21
10 °C	0.09	0.15a	0.26a	0.32a
10 °C + 1 d 25 °C	-	0.10ab	0.06b	0.23b
25 °C	0.09	0.09b	0.06b	0.19b
CV (%)	10.85	28.09	46.82	18.02
F-test	ns	*	*	*

[1] Mean±SE value followed by different letters in the same column area significantly different at $P \leq 0.05$ using DMRT

ns = non-significantly different ($P > 0.05$)

***** = significantly different at $P \leq 0.05$

Appendix Table 26 Internal browning area (%) of Trad-see-thong pineapples treated with GA_3 and stored at 25 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.00	2.94	19.50	76.5
Control	0.00	0.00	0.00	0.00
CV (%)	0	152.13	95.86	38.98
t-test	ns	**	**	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different (P>0.05)

** = significantly different at P≤0.01

Appendix Table 27 Internal browning area (%) of Pattavia pineapples treated with GA_3 and stored at 25 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.00	0.00	20.50	41.50
Control	0.00	0.00	0.00	0.00
CV (%)	0	0	65.94	42.54
t-test	ns	ns	**	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different (P>0.05)

** = significantly different at P≤0.01

Appendix Table 28 PPO activities (units/mg protein) of Trad-see-thong pineapples treated with GA_3 and stored at 25 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.72	0.40	1.60	1.52
Control	0.72	0.71	0.60	0.88
CV (%)	16.34	22.09	7.07	13.59
t-test	ns	*	**	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

***** = significantly different at $0.01<P\leq0.05$

****** = significantly different at $P\leq0.01$

Appendix Table 29 PPO activities (units/mg protein) of Pattavia pineapples treated with GA_3 and stored at 25 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.63	0.54	0.94	1.64
Control	0.63	0.53	0.52	0.93
CV (%)	16.43	8.25	9.70	16.10
t-test	ns	ns	**	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

****** = significantly different at $P\leq0.01$

Appendix Table 30 Endogenous ABA concentrations (µmol/kg FW) in Trad-see-thong pineapples treated with GA_3 and stored at 25 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.20	0.24	0.93	0.24
Control	0.20	0.06	0.27	0.12
CV (%)	22.67	20.07	7.48	5.46
t-test	ns	**	**	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

** = significantly different at $P\leq0.01$

Appendix Table 31 Endogenous ABA concentrations (µmol/kg FW) in Pattavia pineapples treated with GA_3 and stored at 25 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.09	0.16	0.18	0.03
Control	0.09	0.09	0.06	0.19
CV (%)	30.89	25.97	25.48	8.92
t-test	ns	*	*	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

* = significantly different at $0.01<P\leq0.05$

** = significantly different at $P\leq0.01$

Appendix Table 32 Internal browning area (%) of Trad-see-thong pineapples treated with GA_3 and stored at 10 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.00	1.50	9.00	10.00
Control	0.00	6.11	11.67	39.44
CV (%)	0	131.53	48.31	56.79
t-test	ns	ns	ns	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

** = significantly different at $P\leq0.01$

Appendix Table 33 Internal browning area (%) of Pattavia pineapples treated with GA_3 and stored at 10 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.00	0.00	0.00	3.00
Control	0.00	0.00	1.67	9.44
CV (%)	0	0	212.14	122.01
t-test	ns	ns	ns	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

** = significantly different at $P\leq0.01$

Appendix Table 34 PPO activities (units/mg protein) of Trad-see-thong pineapples treated with GA_3 and stored at 10 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.72	0.69	0.38	0.41
Control	0.72	1.10	0.49	0.67
CV (%)	16.43	13.24	23.07	17.58
t-test	ns	**	ns	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

** = significantly different at $P\leq0.01$

Appendix Table 35 PPO activities (units/mg protein) of Pattavia pineapples treated with GA_3 and stored at 10 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.64	0.57	0.61	0.79
Control	0.64	0.64	0.65	0.84
CV (%)	16.34	13.24	8.71	21.66
t-test	ns	ns	ns	ns

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

Appendix Table 36 Endogenous ABA concentrations (µmol/kg FW) in Trad-see-thong pineapples treated with GA_3 and stored at 10 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.20	0.22	0.41	0.17
Control	0.20	0.62	0.71	0.67
CV (%)	22.44	36.08	28.89	30.89
t-test	ns	**	**	**

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

** = significantly different at $P\leq0.01$

Appendix Table 37 Endogenous ABA concentrations (µmol/kg FW) in Pattavia pineapples treated with GA_3 and stored at 10 °C for 21 days.

Treatment	Days in storage[1]			
	0	7	14	21
GA_3	0.09	0.08	0.09	0.03
Control	0.09	0.15	0.26	0.32
CV (%)	42.42	27.51	48.18	15.82
t-test	ns	*	*	*

[1] Asterisks indicate a significant difference between mean values according to student *t*-test

ns = non-significantly different ($P>0.05$)

* = significantly different at $0.01<P\leq0.05$

CURRICULUM VITAE

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