DISCUSSION

1. Effect of low temperatures and maturity stages on pericarp hardening of mangosteen fruit

Pericarp hardening is a common symptom of CI in mangosteen fruit when stored below an optimum temperature (Uthairatanakij and Ketsa, 1996; Choehom *et al.*, 2003). Fruit stored at 6^{o}C had greater pericarp firmness than those stored at 12^{o}C and more mature (reddish purple) fruit had greater firmness than the less mature (reddish brown) fruit (Figure 3). The unacceptable CI symptom of pericarp hardening of mangosteen fruit was found within 5, 10 and 20 days after storage at 3, 6 and 12^{o}C, respectively (Choehom *et al.*, 2003). Shewfelt (1993) reported that CI symptoms might be induced at low temperature, but become prominent upon exposure to non-chilling or high temperatures. This is true also for mangosteen fruit. The present results showed that pericarp hardening was found after storage at 6^{o}C for 9 days and transfer of fruit to room temperature (29.5^{o}C) for 3 days. This confirms the previous studies that pericarp hardening found in mangosteen fruit stored at low temperature and became more prominent upon transfer to high temperatures (Uthairatanakij and Ketsa, 1996; Kosiyachinda, 1986). They did not report the effect of the maturity stages of mangosteen fruit on pericarp hardening after subject to chilling temperature, but found that at the lower temperature the sooner pericarp hardening will develop (Uthairatanakij, 1995).

Severity of CI symptoms in fruit and vegetables depends on many factors including maturity (Von Mollendorff, 1987; Wang, 1990). In general, immature fruit are more sensitive to CI than mature fruit, such as found with avocado (Kosiyachinda and Young, 1976), papaya (Chen and Paull, 1986), tomato (Autio and Bramlage, 1986), mango (Mohammed and Brecht, 2002), persimmon (Salvador *et al*., 2005),

peach (Fernandez-Trujillo *et al*., 1998) and hot pepper seed (Boonsiri *et al*., 2007). In contrast, with mangosteen fruit, it was found that the more mature fruit were more sensitive to CI than less mature fruit. This was similar to that of pineapple fruit that mature fruit developed more blackheart injury than immature fruit. The effect of maturity on this phenomenon was highly correlated to chilling injury symptom of pineapple (Zhou *et al*., 2003).

Pericarp hardening of mangosteen fruit stored at low temperature showed an increase in firmness and lignin contents, while total free phenolics decreased after exposure to low temperature stress (Uthairatanakij and Ketsa, 1996). They showed a negative correlation between total free phenolics and lignin contents and pericarp firmness. This finding was similar to that found in mangosteen fruit after impact (Ketsa and Atantee, 1998; Bunsiri *et al*., 2003). Lignin accumulation was also found in cherimoya fruit after prolonged storage at chilling temperature (Maldonado *et al*., 2002). In another fruit which becomes firmer after harvest, Cai *et al*. (2006a, 2006b) reported that firmness of loquat fruit increased during postharvest ripening and low temperature storage and had a positive correlation with lignin accumulation in the flesh tissue. Lignin is a major plant cell wall component formed through oxidative polymerization of cinnamyl alcohols. It is an important process in higher plants, especially in woody tissues where lignin concentrations reach up to 30% of dry weight. Lignification is a process that also occurs under special conditions such as wounding, pathogen attack or fungal elicitor treatment (Vance *et al*., 1980; Ride, 1983), suggesting a role in plant defense mechanisms.

The degree of pericarp hardening of mangosteen fruit with CI may be related to enzyme activity and phenolics metabolism discussed below. The decrease in phenolic contents occurred concomitantly with increased firmness and lignin contents in mangosteen pericarp stored at low temperature, suggests that phenolics may be

incorporated into lignin (Campbell and Sederoff, 1996; Bunsiri *et al*., 2003) resulting in increased lignin contents and then pericarp hardening. The turnover of phenolics metabolism in mangosteen fruit subject to chilling temperature may be more rapid than its synthesis resulting in decreased phenolics. Total phenolics of many commodities such as loquat, banana, mango and litchi have also been found to decrease during low temperature storage (Ding *et al*., 1998; Nguyen *et al*., 2003, 2004; Ketsa and Chidtragool, 2005; Tian *et al*., 2005; Cai *et al*., 2006a, 2006c). In contrast, storage of jicama roots at 10^{o}C followed by transfer to 20^{o}C induced CI and increased total soluble phenolics (Cantwell *et al*., 2002; Aquino-Bolaños and Mercado-Silva, 2004). Total soluble phenolics also increased in wounded iceberg lettuce (Ke and Saltveit, 1989a, 1989b; Saltveit, 2004), salicylic acid-treated grape berries (Chen *et al.*, 2006) and high O_2-treated blueberry fruit (Zheng *et al*., 2003). Ju and Bramlage (2000) reported that during cold storage, free phenolics in 'Delicious' apple fruit cuticle increased in early storage, and then remained constant. These suggested that turnover of phenolics metabolism is less rapid than their synthesis, resulting in increased phenolics. The difference between the results of these two sets is likely to be in the incorporation of phenolics into lignin in the first set, whereas phenolics accumulation in tissues in response to wounding or elicitors such as salicylic acid are direct responses to disruption of cell compartment with tissue damage or part of defense mechanisms.

At the start of the storage time, total free phenolics in reddish brown fruit were lower than in reddish purple fruit (Figure 5). This was similar to acerola fruit (Lima *et al*., 2005). In contrast to loquat fruit, phenolic compounds in young fruit were high and then decreased steadily during growth (Ding *et al*., 2001). Both of pepper fruit (Estrada *et al.*, 2000) and pepper seeds (Boonsiri *et al*., 2007) also had higher total phenolics at the early development stage. The greater CI symptom in mangosteen pericarp at more mature stage may be due to the higher total phenolics

available as substrates for lignin synthesis. More browning of pepper seeds at younger stage support this idea (Boonsiri *et al.*, 2007). After exposure to CI temperature, total free phenolics decreased and lignin contents increased more rapidly in reddish purple fruit than in reddish brown fruit (Figures 4 and 5). Moreover, changes in total free phenolics and lignin contents in the pericarp of stored mangosteen fruit were greater at lower temperature. These indicated that the incorporation of total free phenolics into lignin synthesis in fruit pericarp might be greater in more mature fruit and at lower temperatures than in less mature fruit and higher temperatures, resulting in the greater pericarp firmness of reddish purple fruit stored at 6^{o}C. Our results found that total free phenolics decreased throughout the storage time whereas PAL activity slightly increased only at the end of storage, suggesting that total phenolics were slightly incorporated into lignin contents at the initial time, while its synthesis started late during storage and the turnover of phenolics metabolism was higher than its synthesis. This also indicated that CI induced PAL activity has a lag period before the PAL activity increased. The late increase in PAL activity and the subsequent lignin accumulation support this idea.

The main phenolic acids in mangosteen pericarp were identified as *p*-coumaric and sinapic acids (Bunsiri *et al.*, 2003). Our present results found that *p*-coumaric acid in reddish purple pericarp stored at 6^{o}C slightly decreased but not in 12^{o}C storage, whereas sinapic acid increased throughout the storage time (Figure 6), suggesting that in the step where *p*-coumaric acid incorporated into other intermediate phenolics including sinapic acid was slow before incorporated finally into lignin. This results in a slight decrease in *p*-coumaric acid levels, and an increase in the accumulation of sinapic acid during low temperature storage. Level of sinapic acid of fruit stored at 6^{o}C was higher than fruit stored at 12^{o}C. This suggested that the accumulation of sinapic acid occurs more rapidly at lower temperature. Other possible reason is lignin synthesis in mangosteen pericarp during

low temperature storage may use other phenolics such as cafeic, ferulic or 5-hydroxyferulic acids as substrate (Whetten and Sederoff, 1995). Phenolics metabolism in mangosteen fruit stored at low temperature may be different from that of pericarp hardening of mangosteen fruit after impact (Bunsiri *et al*. (2003). Bunsiri *et al*. (2003) have demonstrated that both *p*-coumaric and sinapic acids decreased and were incorporated into lignin with pericarp hardening after impact. Since there is more than one lignin synthetic pathway in plants (Whetten and Sederoff, 1995; Campbell and Sederoff, 1996), pericarp hardening of mangosteen fruit at low temperatures may occur through the second synthetic pathway involving the incorporation of both *p*-coumaric and sinapic acids into lignin and possibly the rate of the conversion of *p*-coumaric acid to the *p*-hydroxyphenyl residue may occur more rapidly than that of sinapic acid to the syringyl residue. Furthermore, a cell wall fraction isolated from Vigna epicotyls, which contained bound peroxidases, rapidly oxidized *p*-coumaric, caffeic and ferulic acids and slowly oxidized sinapic acid (Takahama and Oniki, 1994). Hence this would result in a decrease in *p*-coumaric acid and an increase in sinapic acid. However, this needs further study.

The increase on lignin contents on fruit stored at 6^{o}C and transferred to room temperature was substantial when compared to increased pericarp firmness based on the initial time. The increase in pericarp firmness after 6^{o}C storage was about 16-fold, while the increase in lignin contents was about 1.75-fold. This suggested that increased lignin contents alone may not solely contribute to the rapid increase in pericarp firmness after low temperature storage. Lignins in plant cell wall consist of both free and bounded to cell wall polysaccharides (Pearl, 1967). Lignin has been shown to participate in cross-linking between the lignin polymers and cell wall polysaccharides (Kondo *et al.*, 1990; Lam *et al*., 1994; Ralph *et al*., 1995), for example, direct ester linkage between uronic acids and hydroxyl groups on lignins, and ether linkage between polysaccharides and lignins (Iiyama *et al.*, 1994).

Lawoko (2005) reported that lignin is linked through covalent bonds to all the major polysaccharides in the woody cell wall such as arabinoglucuronoxylan, galactoglucomannan, glucomannan and cellulose. Furthermore, linkages were also formed between lignin and proteins (Keller *et al*., 1988; Whetten *et al*., 1998). These may result in strong lignin complexes (Iiyama *et al*., 1994), and then increase in pericarp firmness of mangosteen fruit after low temperature storage.

In plant tissues, lignin synthesis is correlated with activities of many enzymes such as PAL, CAD and POD (Lewis and Yamamoto, 1990). Damaged mangosteen pericarp showed a rapid increase in PAL, CAD and POD activities, with maximum activities occurred about 15 min after impact (Bunsiri, 2003). The increased firmness of loquat fruit (Cai *et al*., 2006b) and bamboo shoot (Luo *et al*., 2007) was a consequence of flesh tissue lignification, and associated with an increase of PAL, CAD and POD activities. PAL is an important enzyme required for synthesis of phenolic compounds that catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid, a precursor of various phenylpropanoids, such as lignins, coumarins and flavonoids (Hahlbrock and Scheel 1989; Lewis and Yamamoto 1990; Schuster and Retey, 1995). Chilling temperatures can generally stimulate the biosynthesis of phenolic compounds by enhancing PAL activity (Aquino-Bolaños *et al*., 2000). PAL activity has been reported to involved with CI development in many plants such as 'Fortune' mandarin, pineapple and 'Navelate' oranges (Martínez-Téllez and Lafuente, 1993; Sanchez-Ballesta *et al*., 2000a; Zhou *et al*., 2003; Sala *et al*., 2005). PAL activity of fresh-cut asparagus also increased in the first 10 days, before decreasing during the latter period of storage concomitant with lignin content (An *et al.*, 2007). These results are similar to findings in the present study on mangosteen fruit stored at 6^{o}C.

Our results from semi-quantitative RT-PCR, real-time PCR and northern analyses showed that PAL mRNA accumulated at the initial of the storage at 6^{o}C and then decreased thereafter. This suggested that PAL gene in mangosteen fruit stored at low temperature was induced at the transcript level prior to the increased PAL activity, lignin accumulation and pericarp hardening. This confirms the reports of Sanchez-Ballesta *et al.* (2000b) that PAL mRNA in 'Fortune' mandarin fruit exposed to 2^{o}C accumulated prior to an increase in PAL activity and then accompanied the exhibition of chilling symptoms. Thus accumulation of PAL mRNA at initial time without increase in PAL activity and phenolic levels might be a common direct low temperature response, while their accumulation in fruit transfer to room temperature was concomitant with the pericarp hardening may result from CI effect.

The main role of POD is attributed to lignification and suberization (Müsel *et al*., 1997; Quiroga *et al*., 2000). This enzyme is required in the final step of lignin synthetic pathway for polymerization of monomeric lignin precursors such as *p*-coumaryl, coniferyl and sinapyl alcohols to form the lignin polymers (Imberty *et al*., 1985; Whetten and Sederoff, 1995; Quiroga *et al*., 2000). POD activity of mangosteen fruit stored at 6^{o}C increased slightly at the initial time of storage and their activity became higher after transfer to room temperature (Figures 16 and 22), resulting the increased lignin content. This was similar to avocado fruit (Hershkovitz *et al*., 2005) and pear flesh (Lee *et al.*, 2006). POD activity in carambola fruit stored at 2^{o}C also increased to a maximum after 25 days before the CI symptom development (Pérez-Tello *et al*., 2001). Our results also found that POD activity in fruit pericarp increased continuously when stored at low temperature more than 12 days (data not shown). This suggests POD activity was induced by low temperature. Increased POD activity in mangosteen fruit after transfer to room temperature was similar to cut jicama (Aquino-Bolaños and Mercado-Silva, 2004) and onion bulbs (Benkeblia, 2000). 'Fortune' citrus fruit treated with 37^{o}C for 3 days (high temperature condition) and then stored at 2.5 and 10^{o}C showed that POD activity in

fruit at high temperature condition was higher than in non-conditioned fruit throughout the storage period (Martinez-Tellez and Lafuente, 1997). These confirm that POD activity was also induced by high temperature in fruit previously stored at low temperature. The results from semi-quantitative RT-PCR, real-time PCR and northern analyses showed that the accumulation of LgPOD mRNA increased at 6^{o}C and after transfer the fruit to high temperature concomitant with increased POD activity, resulting in the increased of lignin contents and the pericarp hardening.

2. Effect of low O_2 on pericarp hardening of mangosteen fruit during and after low temperature storage

Low O_2 treatment applied during and after low temperature storage of mangosteen fruit did not reduce pericarp hardening. Pericarp firmness and lignin contents still increased under low O_2 during storage at 6^{o}C (Figures 11 and 12) and at room temperature after transfer from 6^{o}C (Figures 17 and 18). This was contrast to previous reports that mangosteen pericarp damaged after impact and fruit stored at low temperature under N_2 was less firm, and had lower lignin contents and more total phenolics than damaged pericarp held in the air (Uthairatanakij, 1995; Ketsa and Atantee, 1998; Bunsiri *et al*., 2003). Low temperature may exert more inhibitory effect on many enzymes involved in lignin synthesis, while low O_2 level had effect at last step of monolignol polymerization to complete the process of lignification (Imberty *et al*., 1985). We confirmed this by applying low O_2 treatment to stored mangosteen fruit after transfer to room temperature, and this had a greater effect on pericarp hardening than low O_2 treatment applied only during low temperature storage (Figures 11 and 17). This suggests that low temperature may delay biochemical reactions involved in PAL and POD activities (Figures 14 and 16). Therefore, turnover of phenolic metabolism would be slower. Upon transfer to room temperature, all biochemical reactions involved in lignin synthesis would became more rapid and require more O_2 as well. In addition, only 0.25% O_2 was detected in

the atmosphere under these conditions, but the dissolved O_2 levels in the pericarp tissue may be higher than 0.25% and enough for *de novo* synthesis of lignin under low temperature stress. Low O_2 treatment at 0.25% in air may be enough for lignin synthesis resulting in pericarp hardening of mangosteen fruit.

With northern and semi-quantitative RT-PCR analysis, PAL mRNA accumulation of mangosteen fruit stored in low O_2 was less than that in fruit stored in air. Low O_2 reduced PAL mRNA accumulation and PAL activity, but not lignin contents. In contrast, modified atmosphere packaging treatment ($2\%O_2 + 5\%CO_2$) of bamboo shoots inhibited PAL and POD activities that have been associated with browning and lignification (Shen *et al*., 2006). Butler *et al*. (1990) reported that hypoxic conditions inhibited the accumulation of RNA and protein synthesis associated with wounding in potato tubers, but still accumulated and translated genes that involved in anaerobic metabolism. Anoxia and hypoxia also caused alterations in the profiles of proteins, mRNA and gene expression (Chang *et al*., 2000; Klok *et al.*, 2002). This might be of the reason for the lower PAL mRNA accumulation of mangosteen pericarp in low O_2 storage.

POD activity in fruit pericarp was low when stored at 6^oC under low O_2 level, but its activity became higher after transfer to room temperature (Figure 16B). This might be due to higher level of O_2 and temperatures. Under the condition of low O_2 levels such as coating (Peng and Jiang, 2003; Dong *et al*., 2004) or modified atmosphere packaging (Shen *et al*., 2006) also reduced the activity of POD. POD activity in fruit pericarp after transfer from 6^oC to room temperature under low O_2 level was low, while in normal air was little increased. This might be caused mainly by low temperature. However, low O_2 was also found no effect on POD activity at the end of storage. This confirms the previous idea that low temperature dominated biochemical changes over low O_2 level. Moreover, POD activity in mangosteen fruit stored at 6^oC under low O_2 and transfer to room temperature was high while lignin

content was low. In contrast, POD activity in mangosteen fruit stored at 6°C under normal air was low while lignin content was high after transfer to room temperature (Figures 12 and 16). This indicated that O_2 is required for the oxidation in the final step of polymerization for lignin synthesis in mangosteen pericarp (Whetten and Sederoff, 1995; Bunsiri *et al*., 2003).

CAD activity in fruit pericarp did not change during low temperature storage and even after transfer to room temperature while pericarp firmness increased. This suggested that CAD may not be a rate-limiting step in lignin synthesis of mangosteen pericarp during low temperature storage. CAD activity in damaged pericarp of mangosteen fruit after impact increased 15 min after impact concomitant with an increase in lignin synthesis and CAD activity increased much more than PAL and POD activities (Bunsiri, 2003). The increased lignin contents in loquat fruit (Cai *et al*., 2006b), copper stress treated *Panax ginseng* root (Ali *et al*., 2006) and bamboo shoot (Luo *et al*., 2007) was also associated with CAD activity. Furthermore, CAD activity in transgenic plants (CAD antisense) such as tobacco (Halpin *et al.*, 1994) and poplars (Baucher *et al*., 1996) was reduced, but amount of lignin did not change. Due to CAD catalyzes the reduction of cinnamaldehydes to cinnamyl alcohols in the last step of monolignol synthesis (Boudet, 2000). Thus, this enzyme might not be related to lignin synthesis in the pericarp hardening of mangosteen fruit stored at low temperature. One of possible reason is that there was sufficient CAD activity for lignification to occur in mangosteen pericarp stored at low temperature.

We concluded that mangosteen fruit stored at 6°C had greater pericarp firmness than those stored at 12°C and reddish purple fruit had greater pericarp firmness than the reddish brown fruit. Reddish purple mangosteen fruit stored at 6°C showed an increase in pericarp firmness concomitant with an increase in lignin contents and a decrease in total free phenolics. Fruit stored at 6°C under normal air and low O_2 conditions, with or without transfer to room temperature showed no

significant difference in firmness, lignin and total free phenolics contents. PAL and POD activities in fruit pericarp stored at low temperature increased considerably upon transfer to room temperature, but not CAD activity. Low O_2 treatment had no effect on enzyme activity, but it had slightly effect on mRNA accumulation. Thus, key enzymes of lignin synthesis in pericarp hardening of mangosteen fruit exposed to chilling temperature are probably PAL and POD. Storage and handling regimes to reduce lignification will be difficult to develop, since it was stimulates by low temperature, and the mangosteen fruit do not respond significantly to low O_2 storage.