# RESULTS

**Experiment 1 Study of the physico-chemical changes of mangosteen pericarp stored at low temperature**

1.1 Effect of maturity stages on pericarp hardening of mangosteen fruit during and after low temperature storage

1.1.1 Pericarp firmness

In reddish brown mangosteen fruit stored at 6 and 12$^{o}$C, pericarp firmness did not change until 12 days and then increased sharply thereafter (Figure 3, Appendix Table 1). When fruit were removed at 3 day intervals and transferred to room temperature, pericarp firmness increased rapidly, from 15-120 N (about 8-fold) following 9 days storage (Figure 3A). At 12$^{o}$C, pericarp firmness showed the same trends as those stored at 6$^{o}$C, but the firmness was lower (Figure 3B). Firmness of the more mature fruit (reddish purple stage) was more variable during the first 12 days and then increased rapidly thereafter (Figures 3C and 3D). Upon transfer to room temperature, firmness increased sharply, approximately 11.5-fold (20 to 230 N). Pericarp firmness of fruit stored at 6$^{o}$C was higher than for those stored at 12$^{o}$C and the reddish purple fruit had greater firmness than the reddish brown fruit (Figure 3, Appendix Table 1). Thus pericarp hardening developed more rapidly when fruit were transferred to room temperature and this symptom was more prominent in the advanced maturity stage.

1.1.2 Lignin contents

Lignin contents in reddish brown fruit stored at 6 and 12$^{o}$C were variable but did not change greatly during storage (Figures 4A and 4B). At the end of storage, lignin contents of reddish brown fruit stored at 6$^{o}$C were higher than that of those stored at 12$^{o}$C (Figures 4A and 4B, Appendix Tables 1 and 4). Upon transfer of fruit to room temperature from 6$^{o}$C, lignin contents increased slightly and were higher at the end of storage period, but at 12$^{o}$C the contents were little different whether kept at that temperature or after transfer to room temperature (Figures 4A and 4B). In the more mature fruit, there was slightly increase in lignin contents during storage, although at 6$^{o}$C, the contents declined towards the end of the storage period (Figure 4C). There was also slightly increase in fruit stored at 12$^{o}$C (Figure 4D). After transfer to room temperature, lignin contents were slightly higher than in fruit kept at 6 and 12$^{o}$C (Figures 4C and 4D). When compared between two temperatures and maturity stages, the results showed that lignin contents of fruit stored at 6$^{o}$C were slightly higher than in those stored at 12$^{o}$C and reddish purple fruit had slightly greater increases in lignin contents than reddish brown fruit (Figure 4).

1.1.3 Total free phenolics contents

Total free phenolics of fruit at both maturity stages decreased rapidly during storage at 6 and 12$^{o}$C and after transfer to room temperature (Figure 5). After transfer to room temperature, mangosteen pericarp stored at 6$^{o}$C had a more rapid reduction in total free phenolics than in fruit stored at 12$^{o}$C. When comparing the maturity stages, total free phenolics of the reddish purple fruit decreased more than the reddish brown fruit (Appendix Tables 6 and 7).

The main individual phenolic acids in reddish purple mangosteen fruit stored at both 6 and 12$^{o}$C were identified by HPLC as *p*-coumaric and sinapic acids. *p*-Coumaric acid in fruit stored at 6$^{o}$C decreased, whereas in fruit stored at 12$^{o}$C did not change. At the end of storage, *p*-coumaric acid in fruit stored at 6$^{o}$C decreased more than those stored at 12$^{o}$C (Figure 6A), but there was no significant difference at both temperatures (Appendix Table 8). On the contrary, sinapic acid in fruit stored at 6 and 12$^{o}$C increased throughout the storage time. Fruit stored at 6$^{o}$C had greater sinapic acid content than those stored at 12$^{o}$C (Figure 6B).

### 1.1.4 Histochemical study

The pericarps of non-chilled and chilled reddish purple fruit were embedded, sectioning, and staining with safranin O and toluidine blue. The results showed that lignin accumulation increased with the pericarp hardening. Lignin stained with safranin O is shown as a red color (Figure 7A) and stained with toluidine blue is shown as dark brown (Figure 7B). Lignin distributed all over in both outer and inner pericarp. However, hardening fruit showed more red of safranin O and more dark brown of toluidine blue than non-hardening fruit, indicated that chilling injury induced an accumulation of lignin resulting in pericarp hardening.

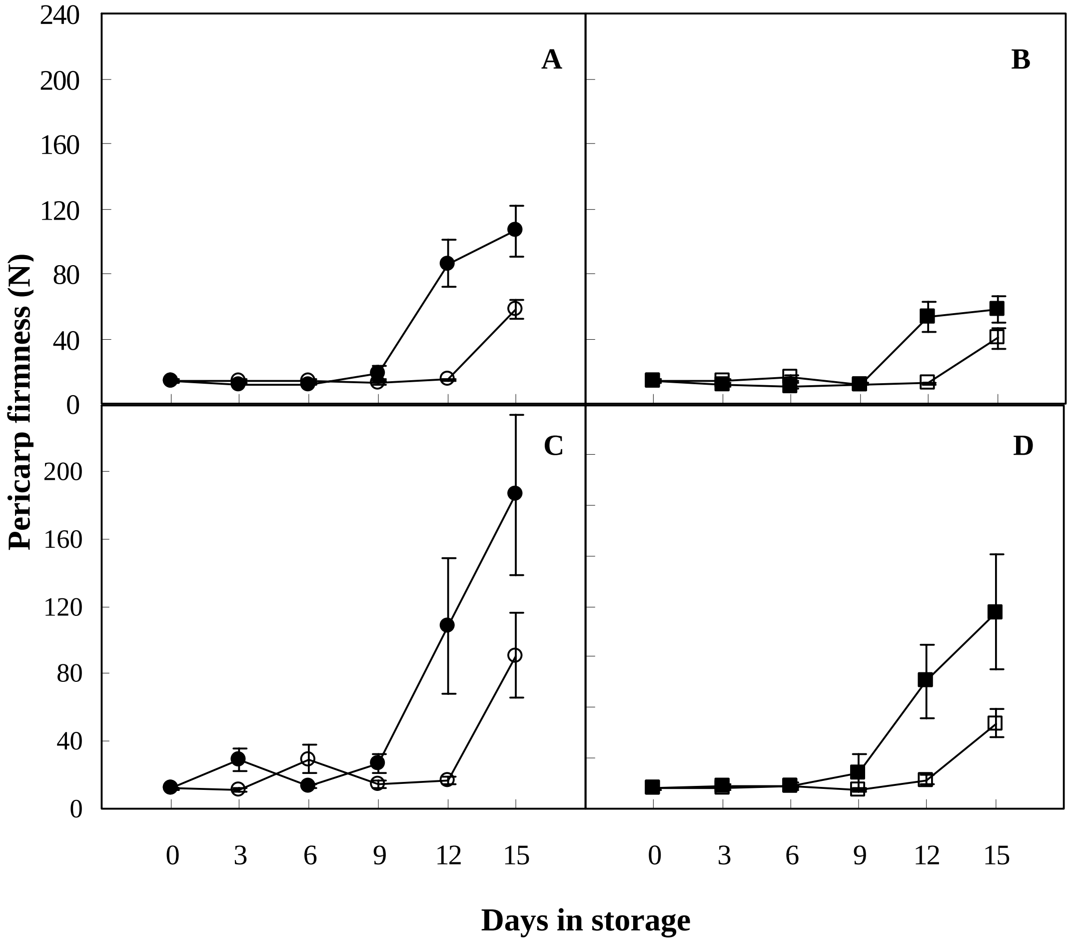


**Figure 3** Pericarp firmness of reddish brown (A and B) and reddish purple (C and D) mangosteen fruit stored at 6°C (○,●) and 12°C (□,■) and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

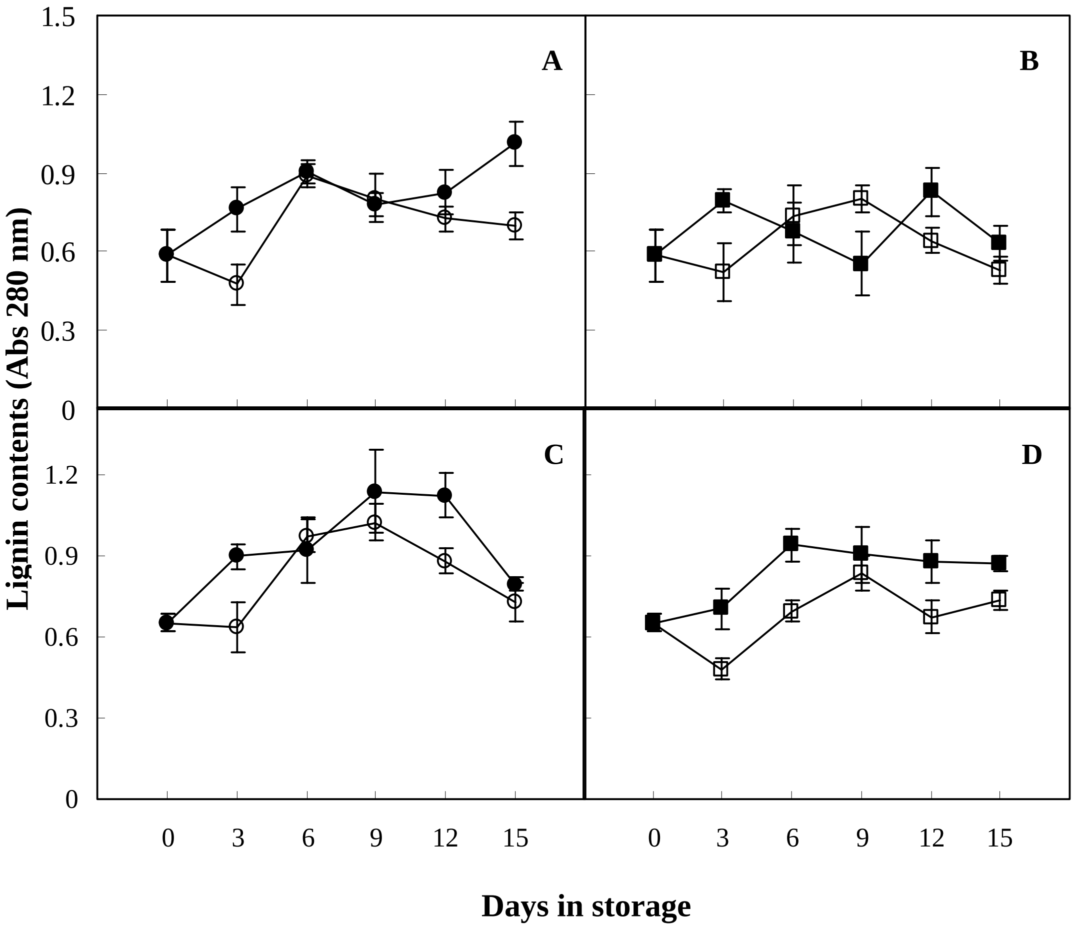


**Figure 4** Lignin contents of reddish brown (A and B) and reddish purple (C and D) mangosteen pericarp stored at 6°C (○,●) and 12°C (□,■) and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

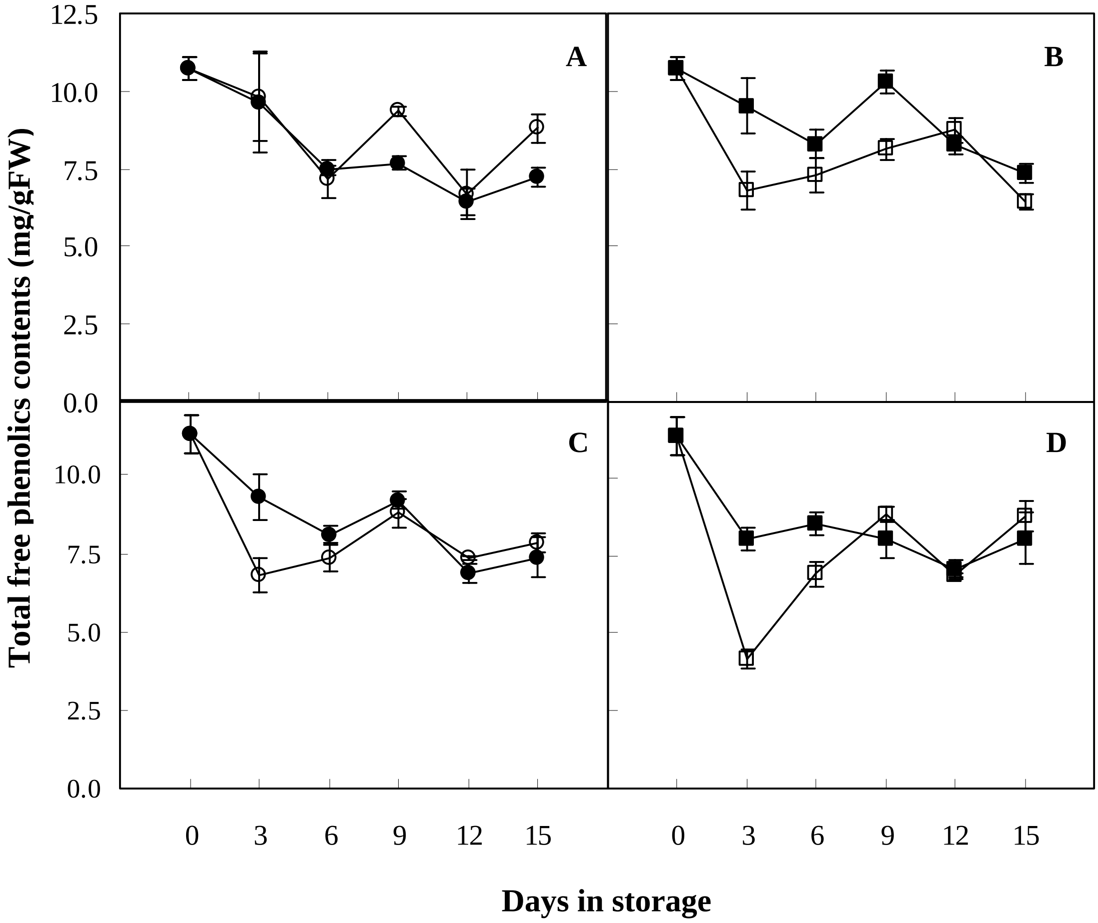


**Figure 5** Total free phenolics contents of reddish brown (A and B) and reddish purple (C and D) mangosteen pericarp stored at 6°C (○,●) and 12°C (□,■) and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

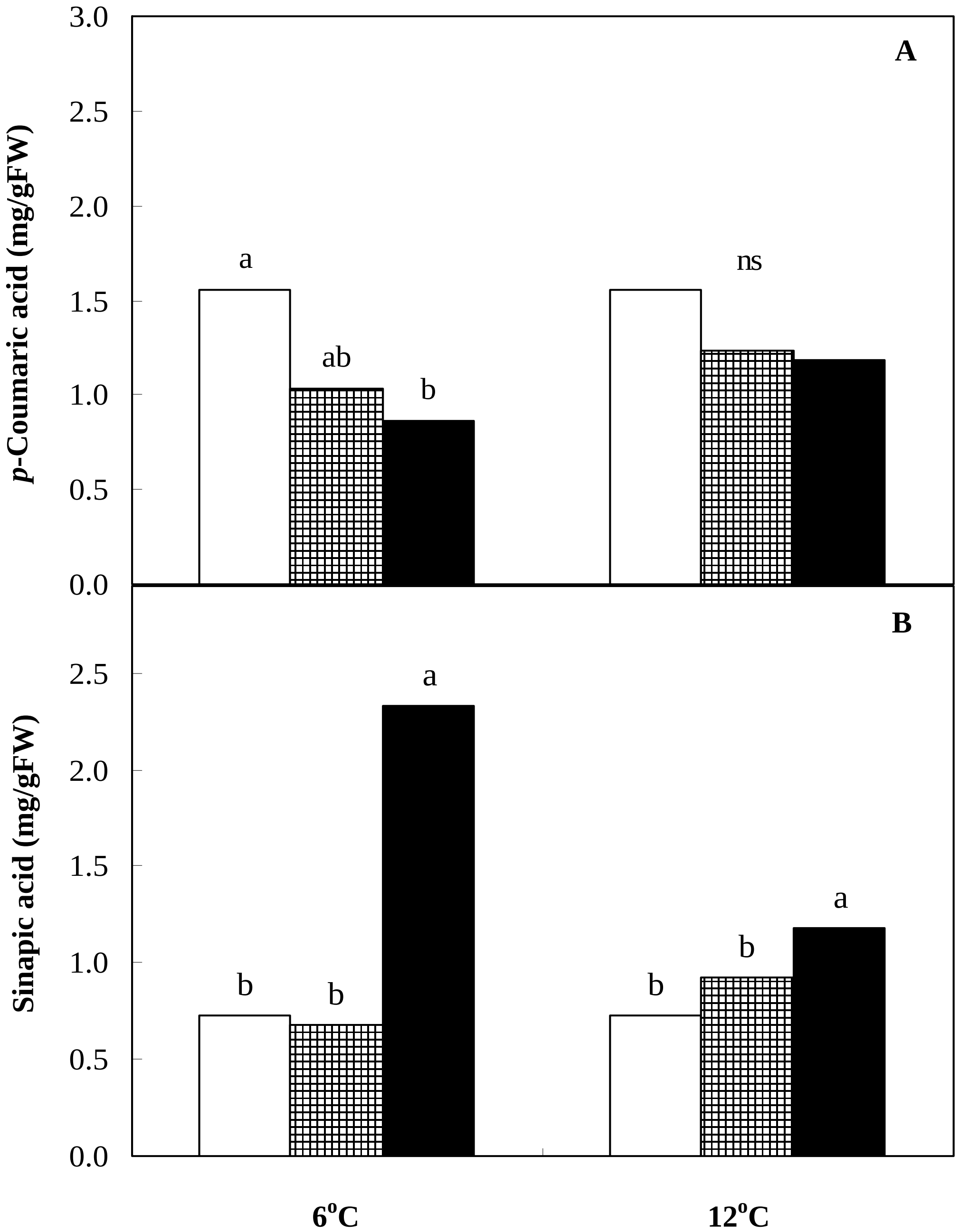


**Figure 6** Changes in (A) *p*-coumaric and (B) sinapic acids of reddish purple mangosteen fruit stored at 6°C and 12°C for 0 (□), 6 (▦) and 12 (■) days. Data are means ± S.D. of three replications.

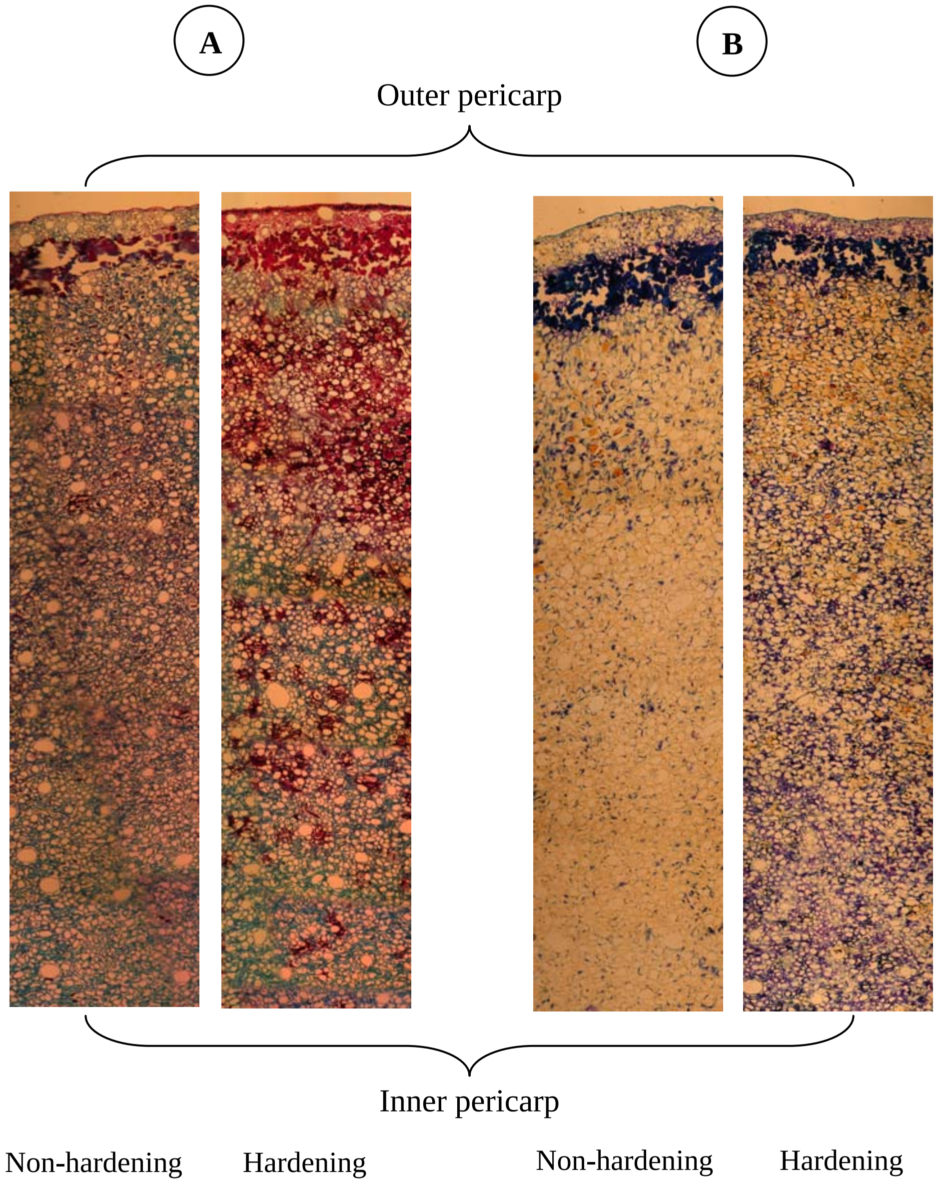


**Figure 7** Non-chilled and chilled pericarp (hardening) of reddish purple fruit stained with (A) safranin O and (B) toluidine blue.

1.2 Effect of low temperature on pericarp hardening of mangosteen fruit after transfer to room temperature

1.2.1 Pericarp firmness

Reddish purple fruit stored at 6$^{o}$C for 0 and 6 days and then transferred to room temperature for 3 days, showed no changes in pericarp firmness (Figure 8). After 12 days storage at 6$^{o}$C, pericarp firmness increased continuously throughout the time at room temperature (Figure 8 and Appendix Table 9).

1.2.2. Lignin contents

Lignin contents in reddish purple fruit after 6$^{o}$C storage remained relatively unchanged (Figure 9). At the end of storage, fruit stored at 6$^{o}$C for 12 days and transferred to room temperature had higher lignin contents than those stored at 6$^{o}$C for 0 and 6 days (Figure 9, Appendix Table 10).

1.2.3. Total free phenolics contents

Total free phenolics contents in reddish purple fruit stored at room temperature did not change, whereas contents in fruit stored at 6$^{o}$C for 6 and 12 days decreased slightly, but there was no significant difference with increasing storage at room temperature (Figure 10, Appendix Table 11).

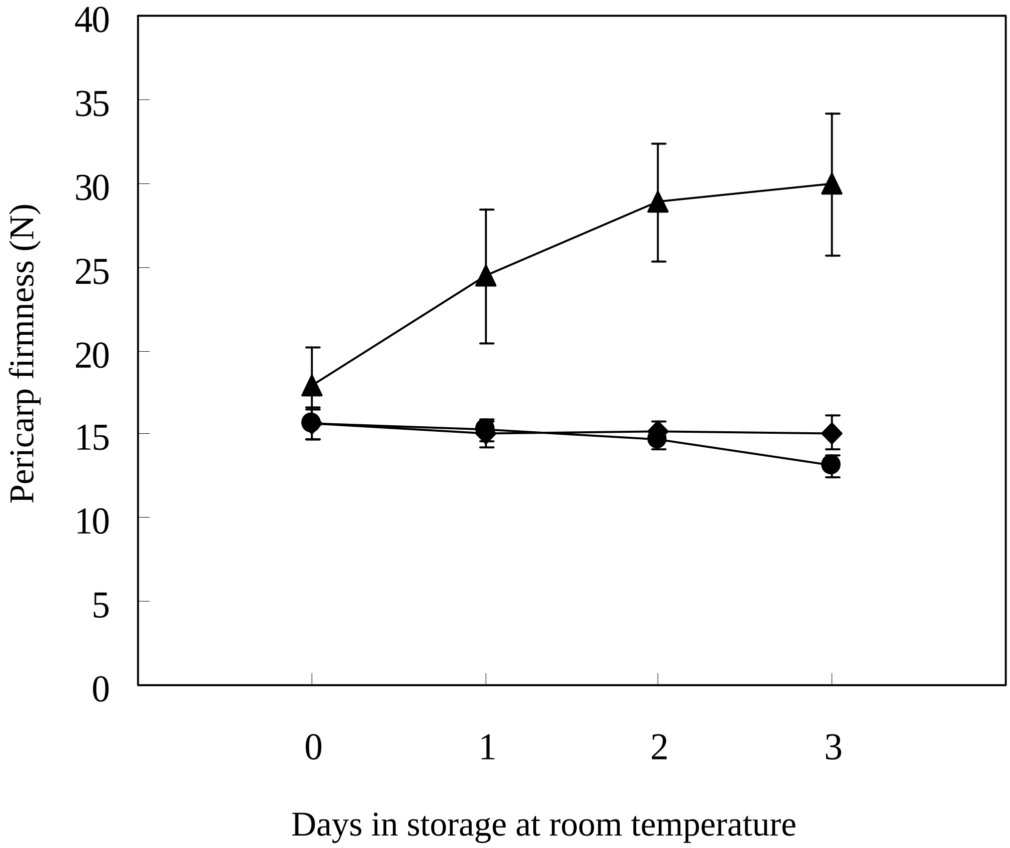


**Figure 8** Pericarp firmness of reddish purple mangosteen fruit stored at 6°C for 0 day (●), 6 days (◆), and 12 days (▲) and transferred to room temperature for 3 days. Data are means ± S.D. of three replications.

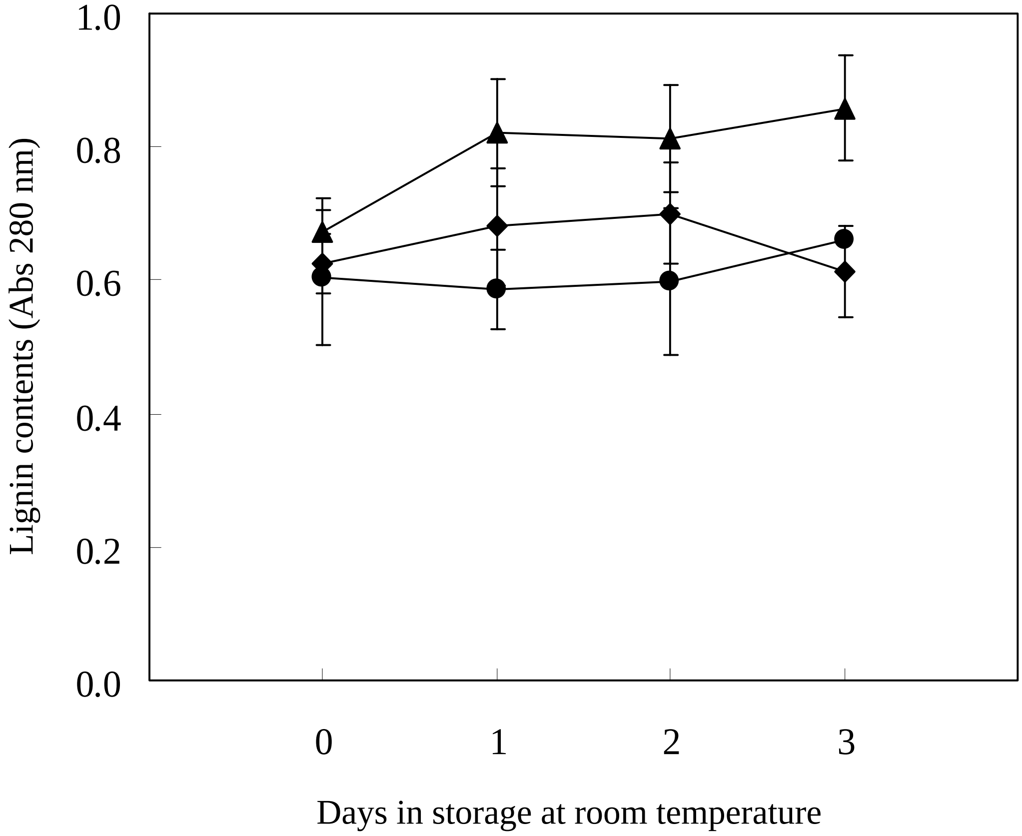


**Figure 9** Lignin contents of reddish purple mangosteen pericarp stored at 6°C for 0 day (●), 6 days (◆), and 12 days (▲) and transferred to room temperature for 3 days. Data are means ± S.D. of three replications.

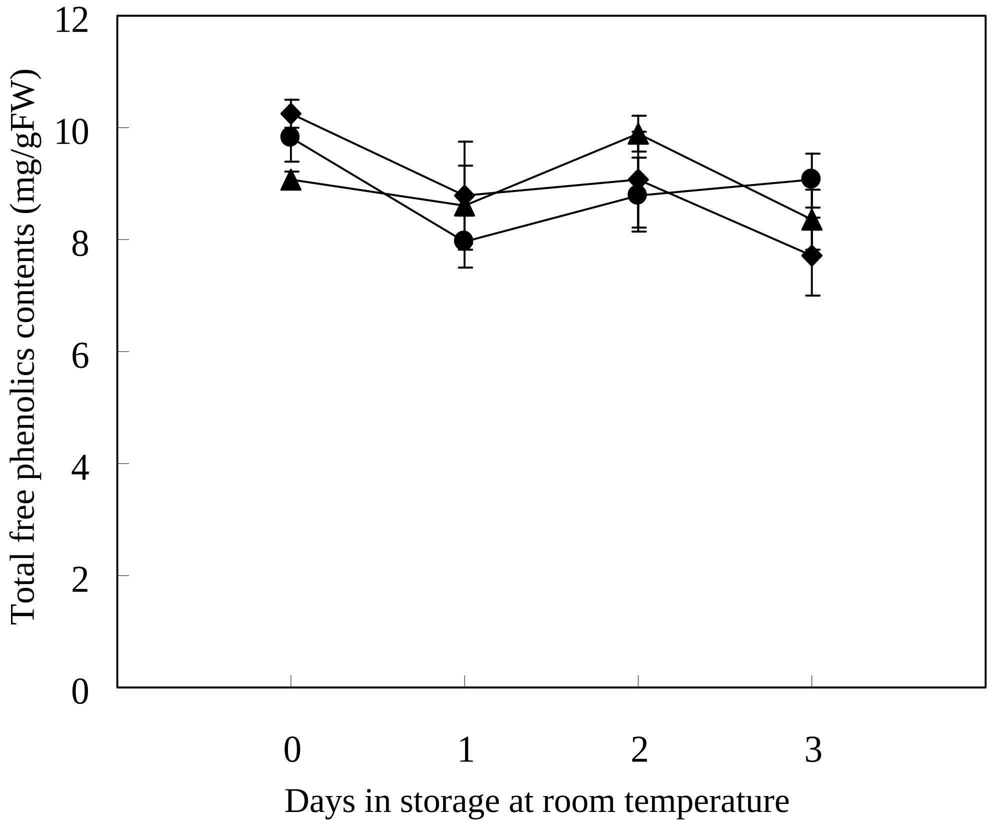


**Figure 10** Total free phenolics contents of reddish purple mangosteen pericarp stored at 6°C for 0 day (●), 6 days (◆), and 12 days (▲) and transferred to room temperature for 3 days. Data are means ± S.D. of three replications.

**Experiment 2 Study on the effect of low $O_2$ on pericarp hardening and some enzyme activities involved in lignin biosynthesis of mangosteen fruit**

2.1 Effect of low $O_2$ on pericarp hardening and some enzyme activities involved in lignin biosynthesis of mangosteen fruit during low temperature storage

From the first experiment, the mangosteen fruit stored at 6°C had greater firmness than those stored at 12°C and reddish purple fruit had greater firmness than the reddish brown fruit (Figure 3). Pericarp hardening of reddish purple fruit stored at 6°C was greater than that of reddish brown fruit and was therefore selected for a study on the effect of low $O_2$ treatment applied during and after low temperature storage.

2.1.1 Pericarp firmness

Pericarp firmness of reddish purple fruit stored under normal air and low $O_2$ conditions at 6°C slightly increased with storage time. When the fruit were removed at 3 day intervals and then held for 3 days at room temperature (30°C, 71.5%RH), pericarp firmness remained unchanged until after 9 days at 6°C and 3 days at room temperature (Figure 11). There were no significant differences between firmness in fruit in normal air and low $O_2$ storage. At the end of storage, pericarp firmness of fruit transferred to room temperature was higher than those of fruit stored at 6°C under both conditions (Figures 11A and 11B, Appendix Table 12).

2.1.2 Lignin contents

Lignin contents in fruit pericarp stored at 6°C did not change much either in normal air or under low $O_2$ conditions. After transfer to room temperature, the contents slightly increased, especially at the end of storage under both conditions (Figure 12, Appendix Table 13). When comparing the treatments, lignin contents of fruit transferred to room temperature were higher than those of fruit stored at 6°C (Figures 12A and 12B).

2.1.3 Total free phenolics contents

Total free phenolics increased slightly in fruit stored at 6°C in both conditions, increasing gradually during the first 6 days and then decreasing at the end of storage (Figures 13A and 13B). There was no significant difference in the data between normal air and low $O_2$ conditions. Phenolic contents of fruit stored at 6°C were higher than those transferred to room temperature and fruit stored in normal were higher than those stored in low $O_2$, but the contents were similar at the end of storage (Appendix Table 14).

2.1.4 Enzyme activities in lignin biosynthesis pathway

PAL activity in fruit pericarp stored at 6°C did not change much (Figure 14). After transfer to room temperature, PAL activity in fruit stored in normal air increased more sharply on day 9 than under low $O_2$, although the data showed a high level of variability (Appendix Table 15).

CAD activity in fruit pericarp decreased with storage time under all conditions, and showed no significant differences between normal air and low $O_2$ storage (Figures 15, Appendix Table 16).

POD activity in fruit pericarp stored at 6°C increased more rapidly in normal air than in low $O_2$, whereas the fruit stored in low $O_2$ showed little change (Figures 16A and 16B). After transfer to room temperature, POD activity in fruit pericarp stored in normal air slightly increased, while that in pericarp stored in low $O_2$ increased sharply on day 6 and decreased thereafter (Figure 16). At the end of storage, there was no significant difference between normal air and low $O_2$ storage activity after transfer to room temperature and POD activity in fruit transferred to room temperature was higher than those stored at 6°C (Appendix Table 17).

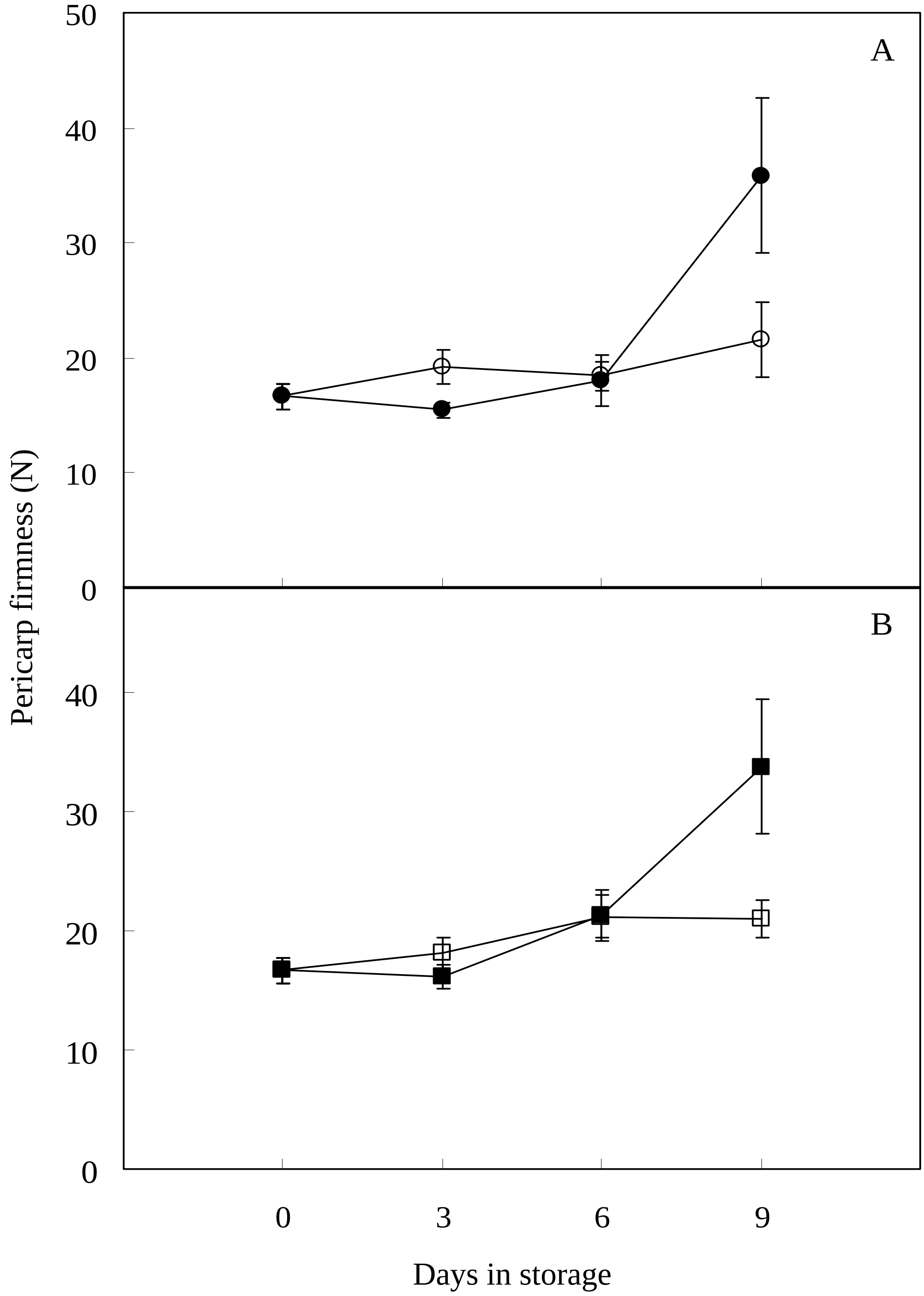


**Figure 11** Pericarp firmness of reddish purple mangosteen fruit stored at 6$^{o}$C in (A) normal air (○,●) and (B) low $O_2$ (□,■) conditions and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means $\underline{+}$ S.D. of three replications.

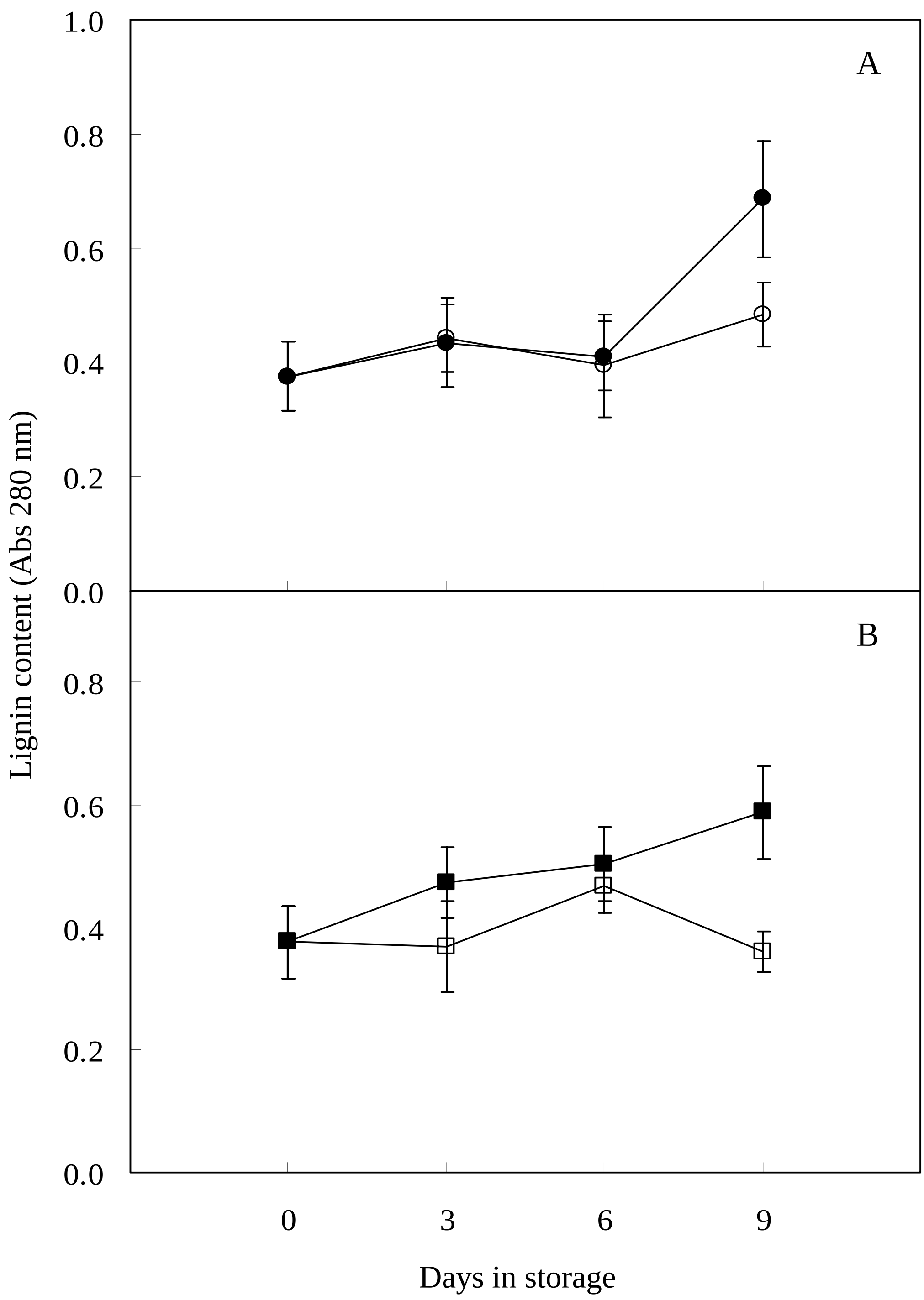


**Figure 12** Lignin contents of reddish purple mangosteen pericarp stored at 6°C in (A) normal air (○,●) and (B) low $O_2$ (□,■) conditions and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

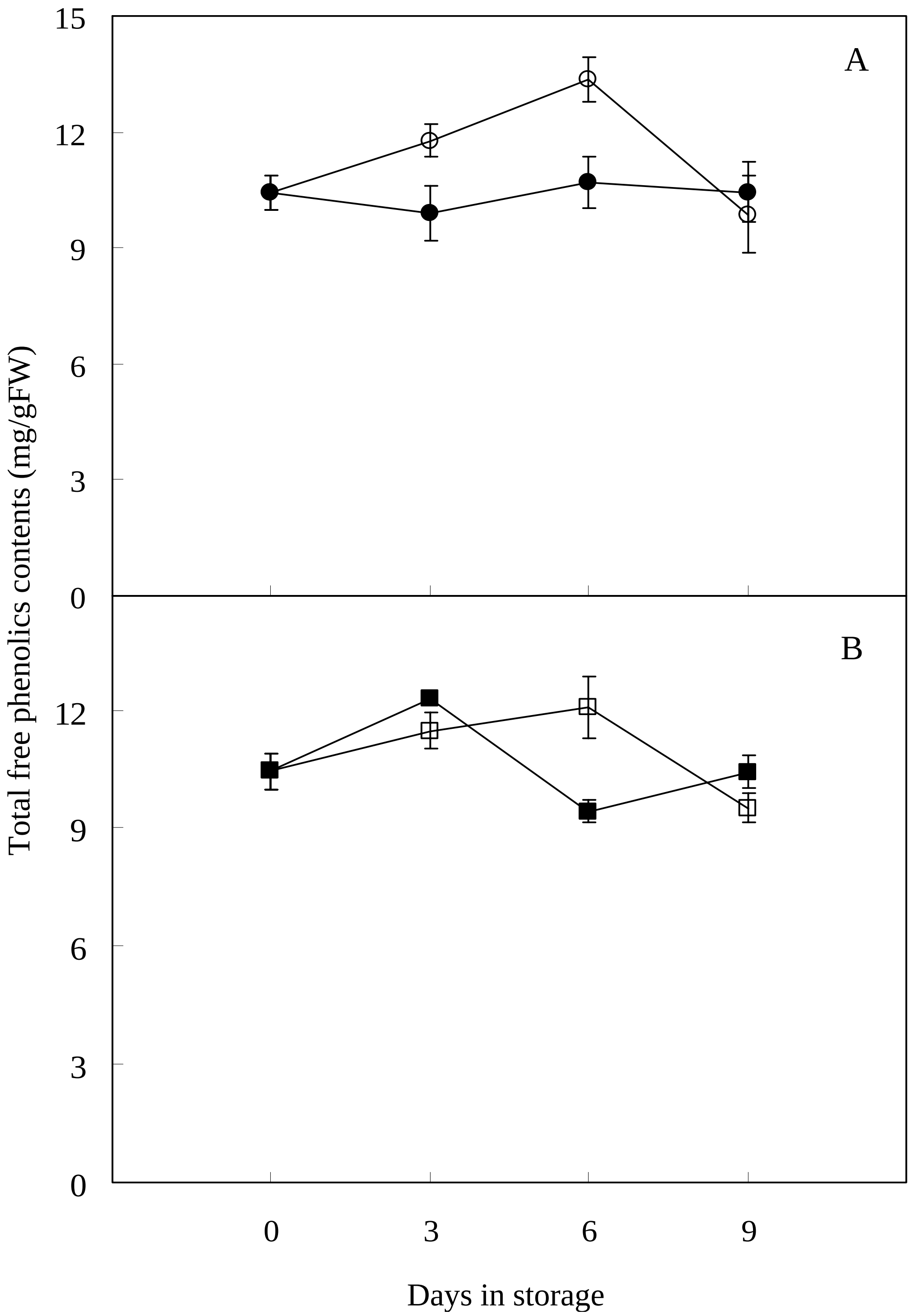


**Figure 13** Total free phenolics contents of reddish purple mangosteen pericarp stored at 6°C in (A) normal air (○,●) and (B) low $O_2$ (□,■) conditions and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

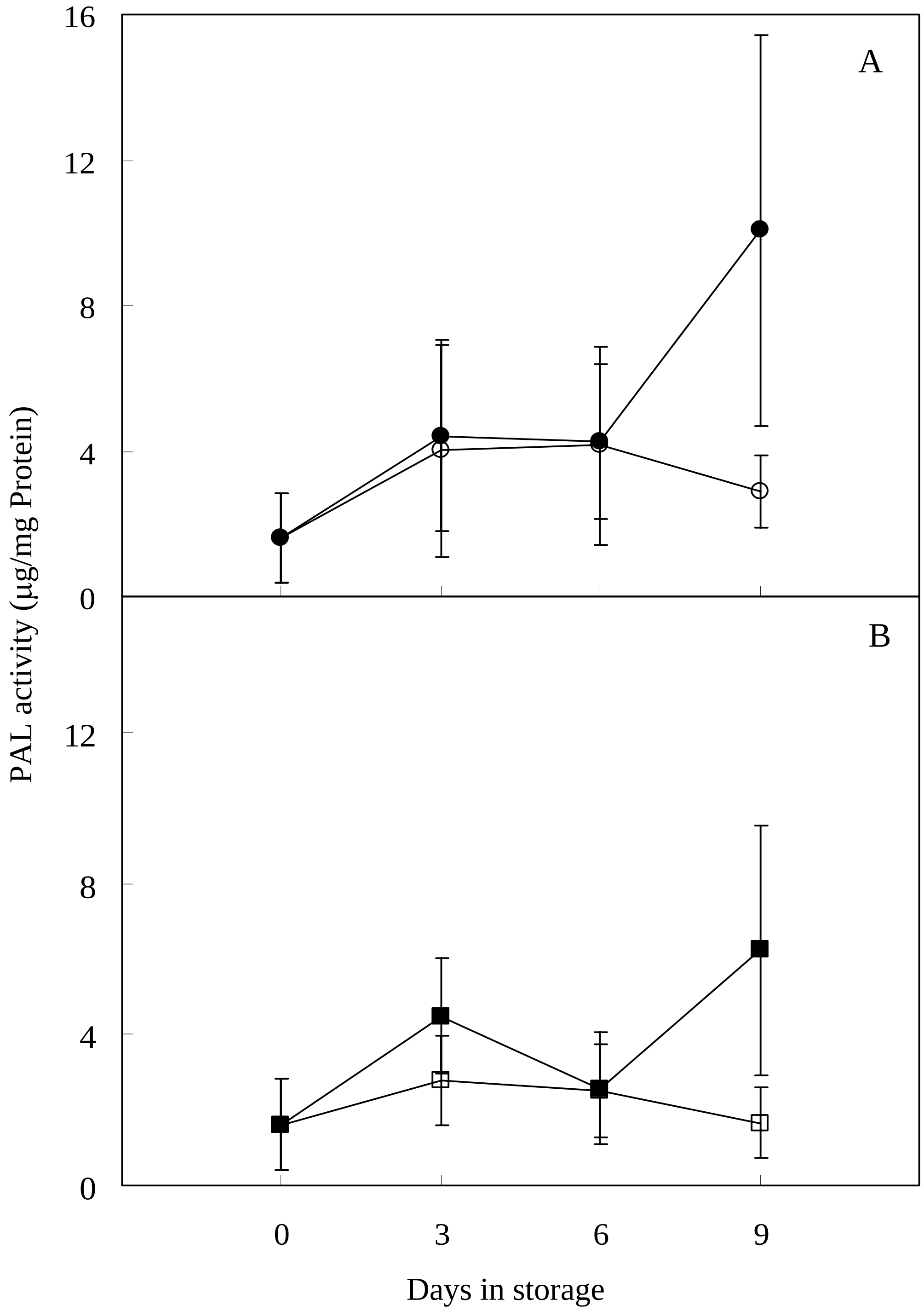


**Figure 14** Phenylalanine ammonia lyase (PAL) activity of reddish purple mangosteen pericarp stored at 6$^{o}$C in (A) normal air (○,●) and (B) low $O_2$ (□,■) conditions and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

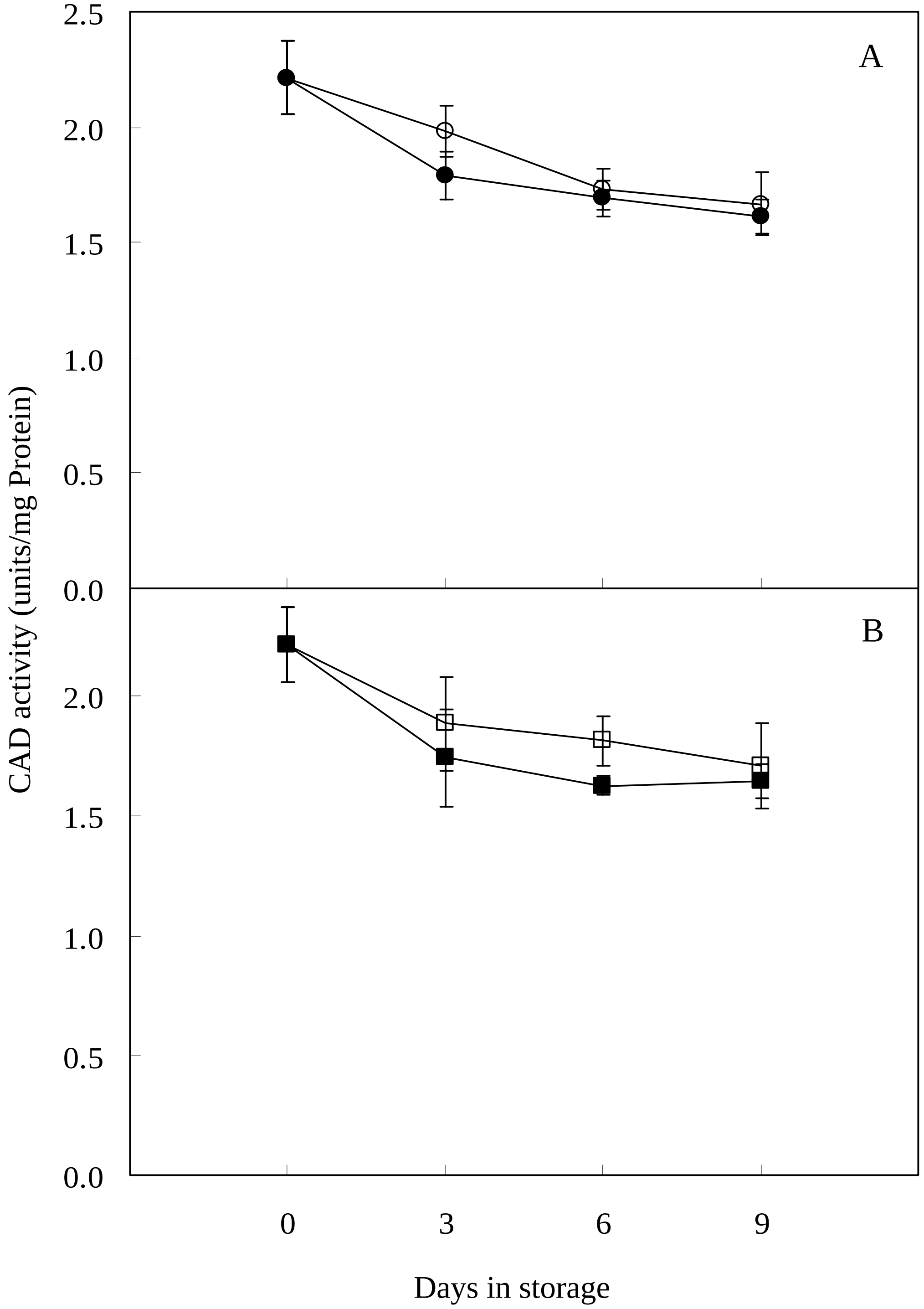


**Figure 15** Cinnamyl alcohol dehydrogenase (CAD) activity of reddish purple mangosteen pericarp stored at 6$^{o}$C in (A) normal air (○,●) and (B) low $O_2$ (□,■) conditions and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means $\pm$ S.D. of three replications.

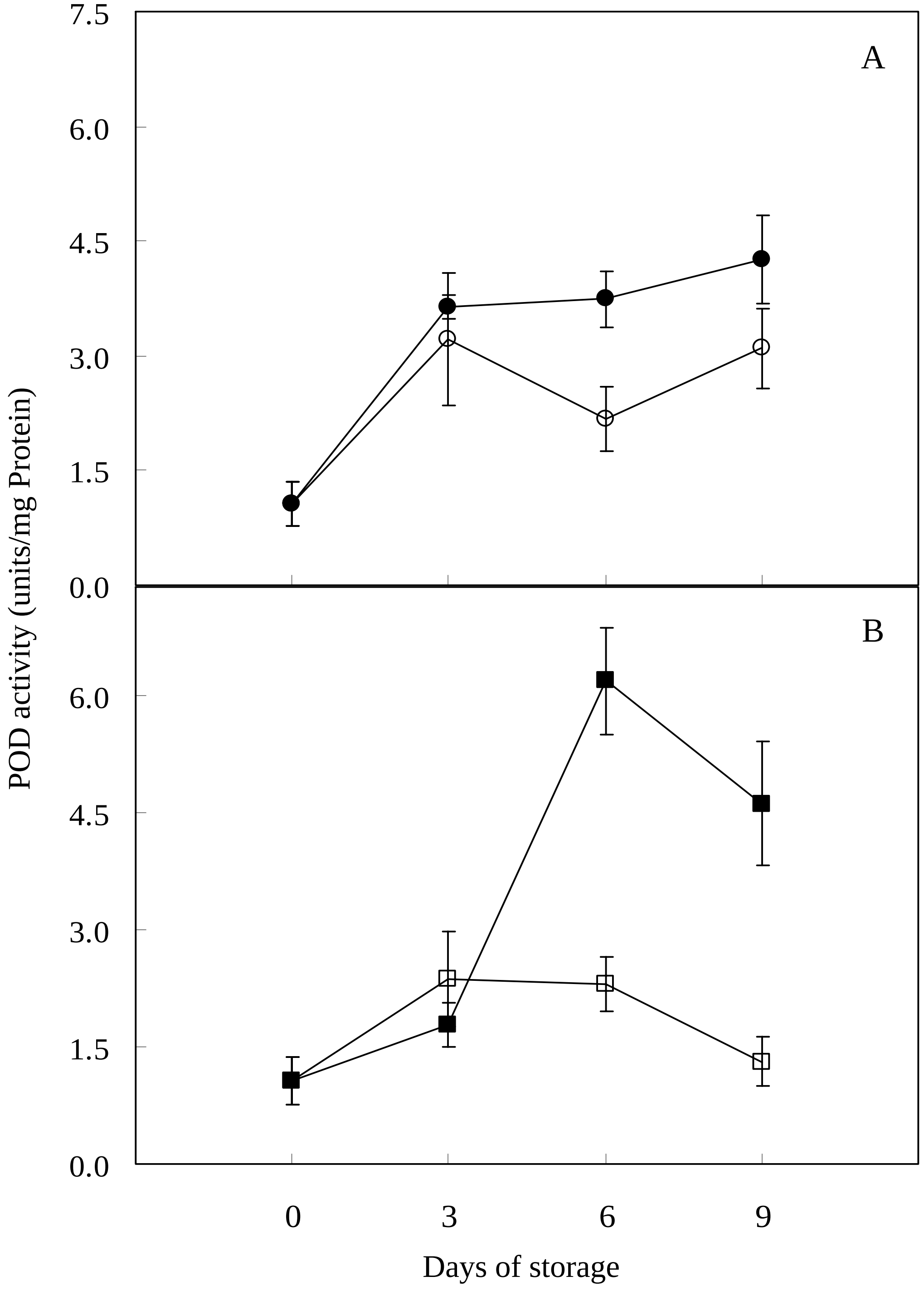


**Figure 16** Peroxidase (POD) activity of reddish purple mangosteen pericarp stored at 6°C in (A) (○,●) and (B) low $O_2$ (□,■) conditions and measured immediately (○,□) and after transfer to room temperature for 3 days (●,■). Data are means ± S.D. of three replications.

2.2 Effect of low $O_2$ treatment on pericarp hardening and some enzyme activities involved in lignin biosynthesis of mangosteen fruit after low temperature storage

2.2.1 Pericarp firmness

This experiment was conducted to investigate the effect of low $O_2$ treatment at room temperature (30$^{o}$C, 71.5%RH), after transfer of fruit from cold storage (6$^{o}$C, 87%RH). Pericarp firmness in reddish purple fruit increased when stored at 6$^{o}$C for 9 days. Upon transfer to room temperature, pericarp firmness increased sharply and showed no significant difference between normal air and low $O_2$ conditions (Figure 17, Appendix Tables 18 and 19).

2.2.2 Lignin contents

Lignin contents of pericarp increased rapidly when fruit were stored at 6$^{o}$C. After transfer to room temperature, lignin contents slightly increased under both conditions but they were no significant difference between normal air and low $O_2$ conditions (Figure 18, Appendix Tables 18 and 20).

2.2.3 Total free phenolics contents

Total free phenolics contents in mangosteen pericarp stored at 6$^{o}$C for 9 days increased slightly (Figure 19). They decreased after transfer of the fruit to room temperature for 2 days and then increased slightly. There was no significant difference in data between normal air and low $O_2$ conditions on the first few days after transfer to room temperature, but then total phenolics in low $O_2$ storage were higher than those in fruit stored in normal air (Figure 19, Appendix Tables 18 and 21).

### 2.2.4 Enzyme activities in lignin biosynthesis pathway

PAL activity increased slightly in the reddish purple mangosteen fruit pericarp after storage at 6$^{o}$C for 9 days. After transfer to room temperature, activity in fruit pericarp stored in normal air increased sharply to a maximum on day 2, and then decreased thereafter (Figure 20). PAL activity in fruit pericarp stored in low $O_2$ conditions for 2 days was lower than that in fruit stored in normal air and there was no significant difference thereafter (Appendix Tables 18 and 22).

CAD activity in fruit pericarp stored at 6$^{o}$C for 9 days and transferred to room temperature for 3 days did not change (Figure 21, Appendix Tables 18 and 23).

POD activity in fruit pericarp stored at 6$^{o}$C increased slightly. After transfer to room temperature, activity in the fruit pericarp stored in normal air was higher than that in fruit stored in low $O_2$ conditions during the first 2 days. At the end of storage (day 3), POD activity slightly decreased and showed no significant difference between normal air and low $O_2$ conditions (Figure 22, Appendix Tables 18 and 24).

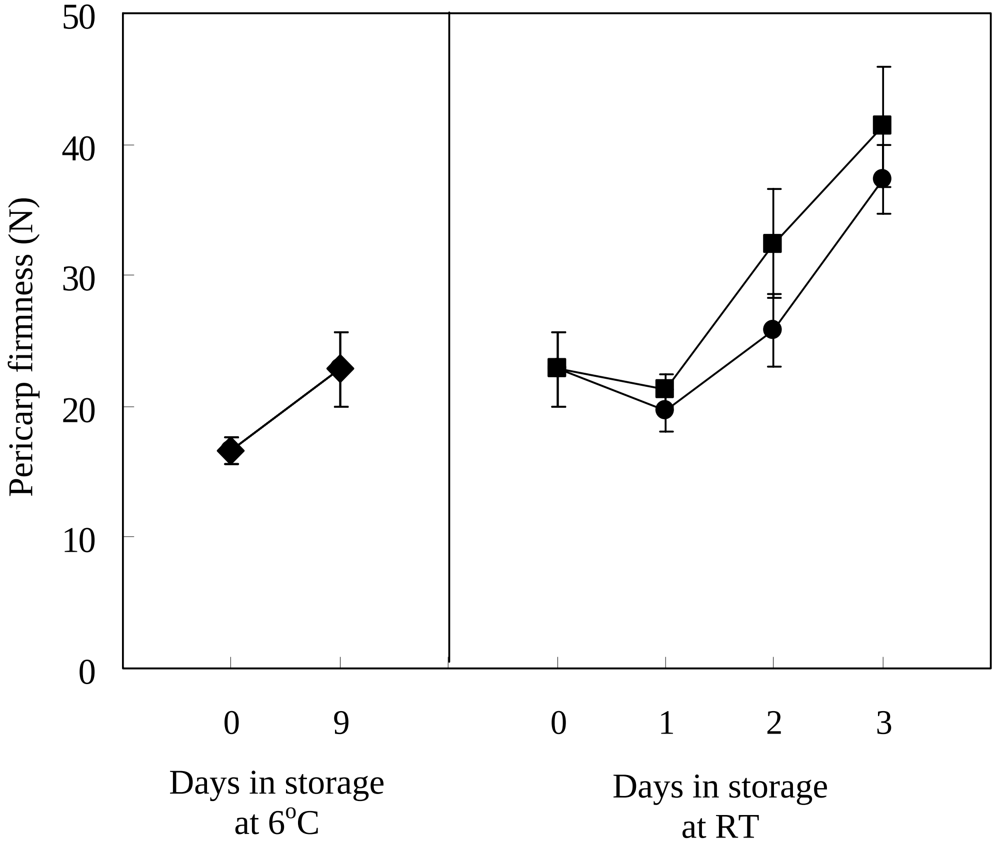


**Figure 17** Pericarp firmness of reddish purple mangosteen fruit stored at 6°C for 9 days (◆) and transferred to room temperature for 3 days in normal air (●) and low $O_2$ (■) conditions. Data are means ± S.D. of three replications.

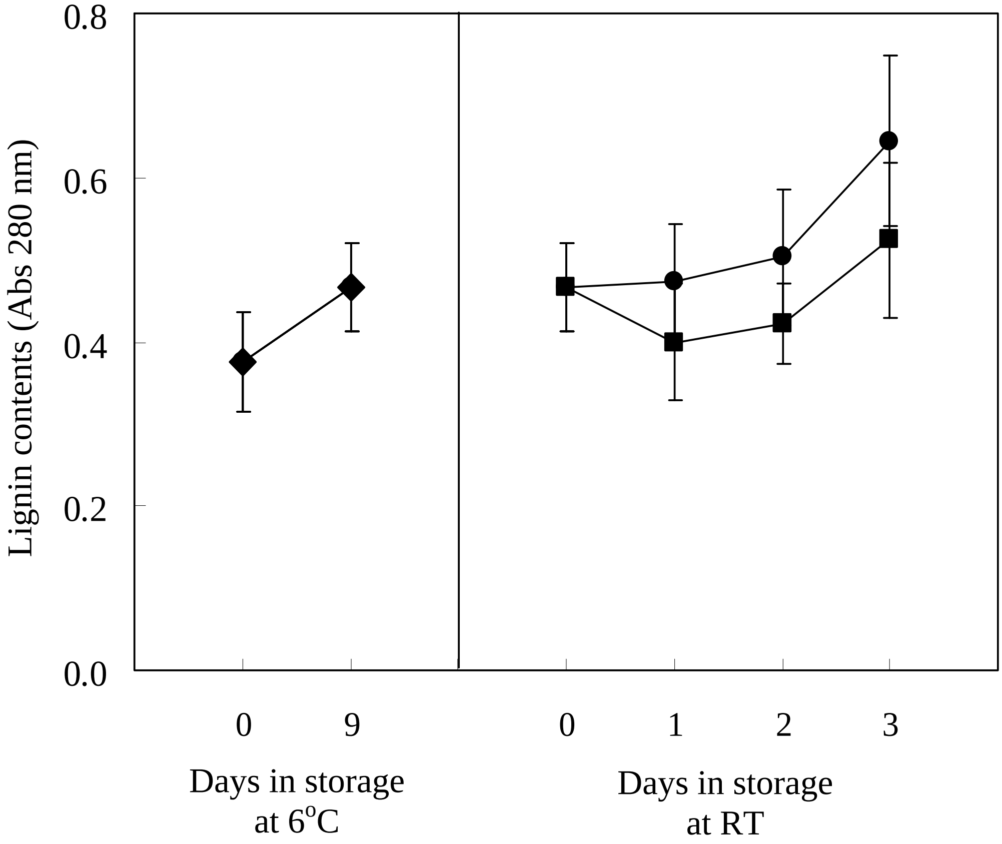


**Figure 18** Lignin contents of reddish purple mangosteen pericarp stored at 6°C for 9 days (◆) and transferred to room temperature for 3 days in normal air (●) and low $O_2$ (■) conditions. Data are means ± S.D. of three replications.

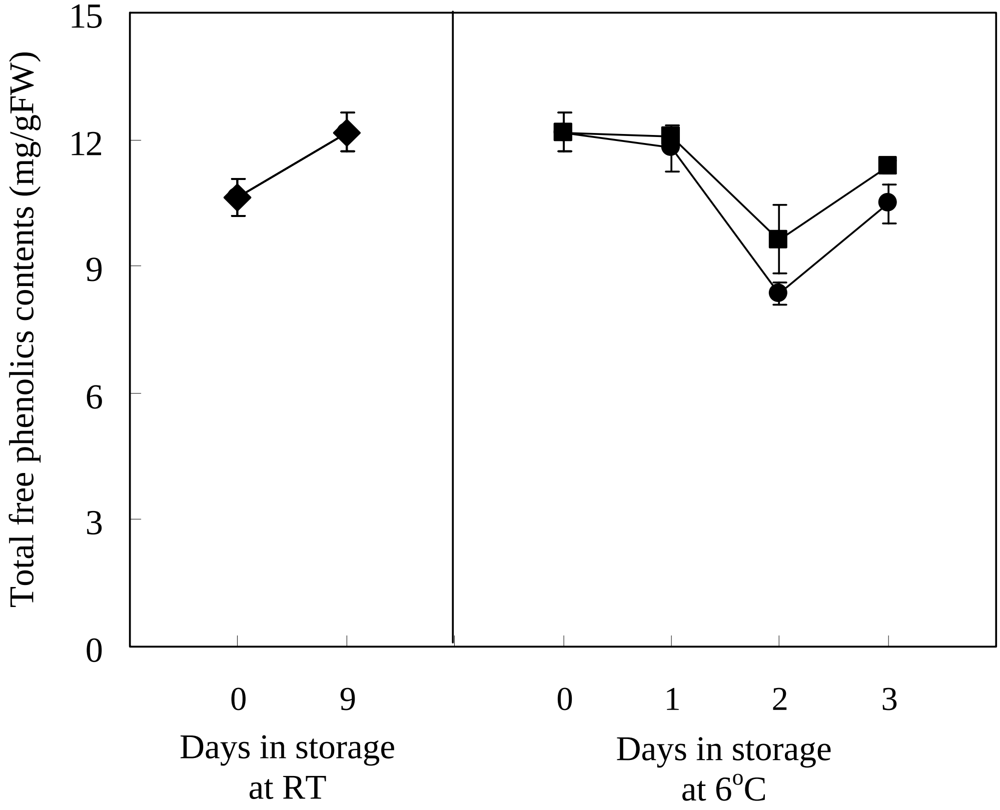


**Figure 19** Total free phenolics contents of reddish purple mangosteen pericarp stored at 6°C for 9 days (◆) and transferred to room temperature for 3 days in normal air (●) and low $O_2$ (■) conditions. Data are means ± S.D. of three replications.

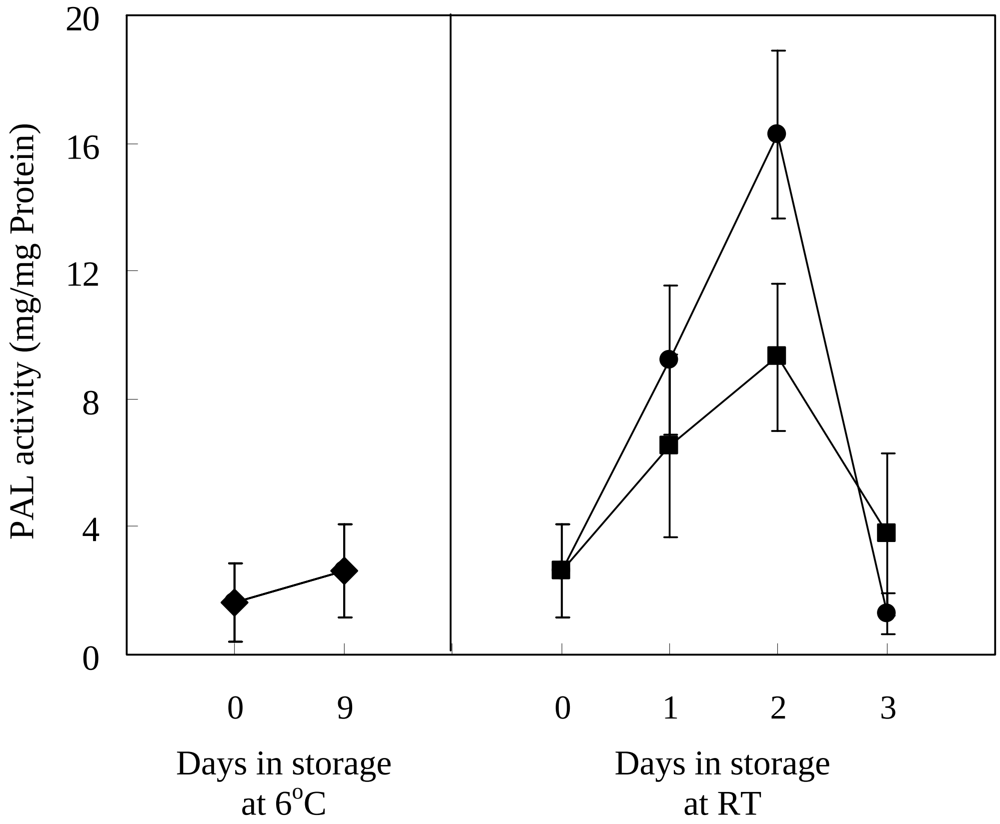


**Figure 20** Phenylalanine ammonia lyase (PAL) activity of reddish purple mangosteen fruit stored at 6°C for 9 days (◆) and transferred to room temperature for 3 days in normal air (●) and low $O_2$ (■) conditions. Data are means ± S.D. of three replications.

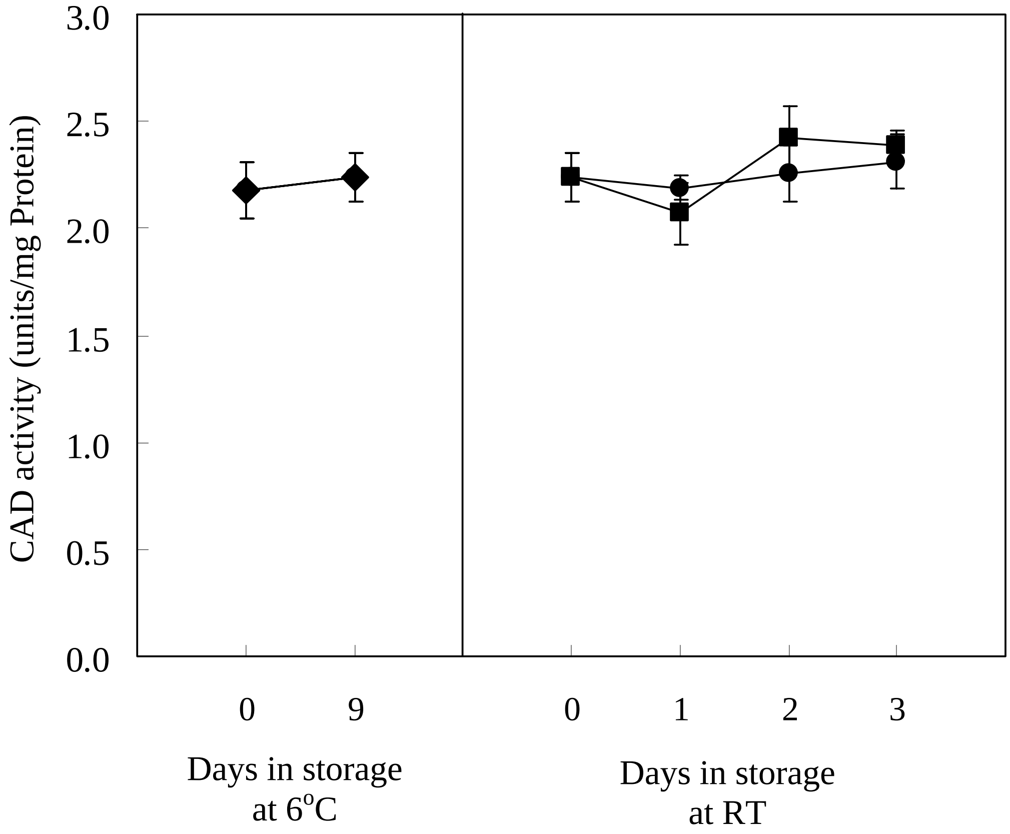


**Figure 21** Cinnamyl alcohol dehydrogenase (CAD) activity of reddish purple mangosteen fruit stored at 6°C for 9 days (◆) and transferred to room temperature for 3 days in normal air (●) and low $O_2$ (■) conditions. Data are means ± S.D. of three replications.

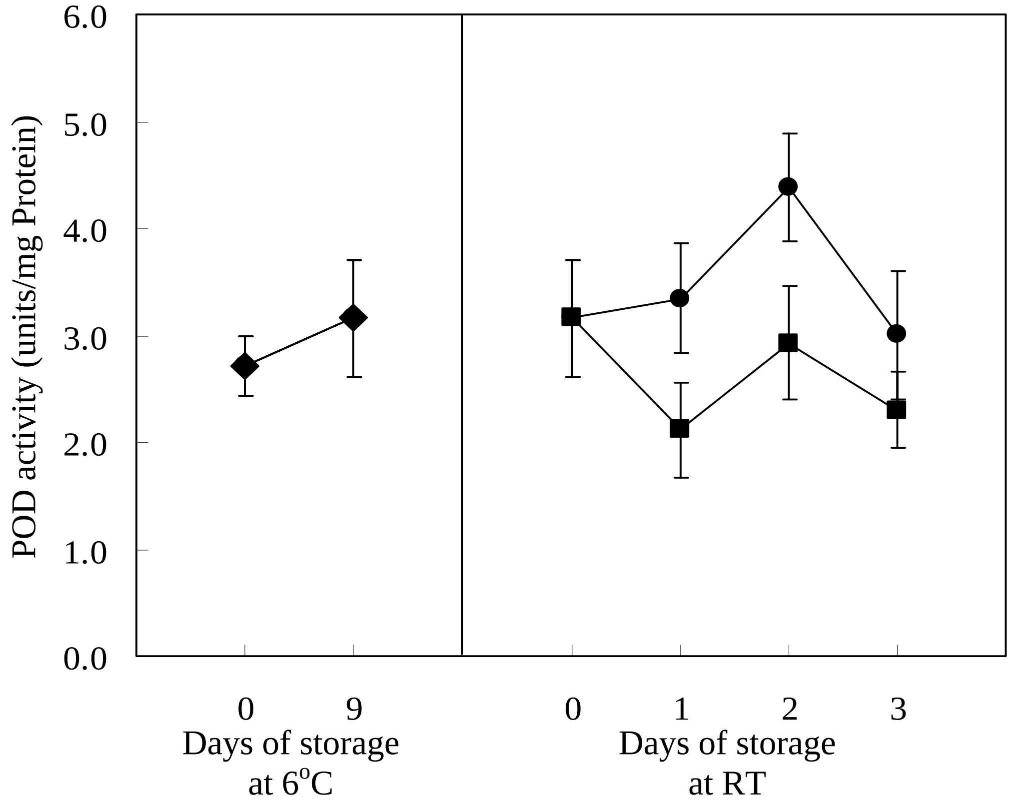


**Figure 22** Peroxidase (POD) activity of reddish purple mangosteen fruit stored at 6°C for 9 days (◆) and transferred to room temperature for 3 days in normal air (●) and low $O_2$ (■) conditions. Data are means ± S.D. of three replications.

2.3 Study of expression of genes associated with the lignin biosynthetic pathway involved in pericarp hardening of mangosteen fruit

The sequences of PAL, LgPOD, 18S rRNA and GAPDH genes in mangosteen are given in Table 6. The bold letter with underline represents forward and reverse primers for product amplification.

**Table 6** The sizes of amplified cDNA fragments and the sequences of genes involved in lignin biosynthesis in mangosteen pericarp

| Gene | Product size (bp) | Sequence |
|---|---|---|
| PAL | 467 | **GTAATTTTCGCAGAGGTTATG**AATGGTAAAGCAGAGTTCA<br>CTGACCACTTGACTCACAAATTGAAGCACCATCCAGGCCA<br>GATTGAGGCTGCAGCTATAATGGAGCACATTCTTGATGGA<br>AGCTCTTATATGAAAGAAGCCAAGAGATTGCATGAGATGG<br>ATCCCTTGCAGAAGCCTAAACAAGATCGATACGCTCTCAG<br>GACTTCACCTCAATGGCTCGGCCCACCTATTGAAGTCATC<br>CGTTTCGCTACAAAAATGATTGAAAGGGAGATTAACTCTG<br>TGAATGACAATCCTTTGATTGATGTTTCCAGGAATAAGGC<br>ATTACACGGTGGCAACTTCCAGGGAACCCCAATTGGAATG<br>TCAATGGACAATGCAAGATTGGCTATTGCTGCTATTGGAA<br>AACTCATGTTTGCTCAATTCAGTGAGCTTGTTAATGACTA<br>CTA**CAACAATGGCTTACCATCAAATCT** |
| LgPOD | 464 | **CATTTTCACGATTGCTTTGTCAACGGTTGTG**ATGGCTCGG<br>TTTTGTTAGATGGCACAGACAGTGAACAAAATGCACAACC<br>GAATCAAGCTCTTGGTGGATTTTCGGTCGTTGATGACATT<br>AAAACAGCGCTTGAGACTTCTTGCCCTGGCGTCGTTTCTT<br>GCGCTGATATACTAGCTTTGGCAGCTGAAATAATGGTTTC<br>TATGGCAGGAGGTCCAACATGGAACGTGCAACTAGGGAGA<br>AGAGATGGATTGAGTGCAAATTCAGCAGGAACAACTGCGA<br>TCCCACTTTCTTCTGATACCCTTGACATCCTCACTGAAAA<br>ATTTTCTGCCAAGGGACTTGATACCACCGATCTAGTTGCT<br>TTATCAGGGGCACATACATTTGGTGTTGCAAGGKGTATAG<br>CATTCAGCAACCGTCTCTACACATTCAACAACACCGAAAA<br>C**CCTGACCCAACTATCAACACAAC** |

**Table 6** (Continued)

| Gene | Product size (bp) | Sequence |
|---|---|---|
| 18S rRNA | 512 | **CGGGGAGGTAGTGACAATAAATA**ACAATACCGGGCTCTTT<br>GAGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAA<br>CGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGC<br>GGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCA<br>GTTAAAAAGCTCGTAGTTGGACCTTGGGATGAGATGATTG<br>GTCCGCCTTTTGGTGTGCACCTGTCATCTCGTCCCTTCTA<br>CCGGCGATGCGCTCCTGGCCTTAATTGGCCGGGTCGTGCC<br>TTCGGTGCTGTTACTTTGAAGAAATTAGAGTGCTCAAAGC<br>ATGCCTACGCTCTGGATACATTAGCATGGGATAACATCAT<br>AGGATTTCGATCCTATTCTGTTGGCCTTCGGGATCGGAGT<br>AATGATTAACAGGGACAGTCGGGGGCATTCGTATTTCATA<br>GTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACAAC<br>TGCGAAAGC**ATTTGCCAAGGATGTTTTCATTA** |
| GAPDH | 409 | **GGGTACAATGACAACTACTCACTCTT**ACACCGGTGATCAG<br>AGGCTACTTGATGCTAGCCACCGCGATCTCCGAAGAGCAA<br>GAGCAGCTGCTCTCAACATTGTCCCTACTTCAACTGGTGC<br>AGCAAAGGCAGTTGCCCTTGTCCTTCCAACTCTAAAGGGA<br>AAACTCAACGGCATTGCCCTTCGTGTACCCACCCCGAACG<br>TGTCTGTTGTTGATCTTGTTGTCCAAGTTTCCAAGAAGAC<br>ATTTGCCGAAGAGGTGAACGCTGCCTTCAGAGAGAGTGCC<br>GAGAAGGAGCTCTCTGGAATACTTTCCGTGTGCGACGAGC<br>CCCTTGTTTCAGTTGACATCAGGTGTACCGATGTTTCGTC<br>AACGGTGGATGCATCACTGACCAT**GGTCATGGGAGATGAT<br>ATGGTTAAG** |

The sequences of PAL and LgPOD genes were translated to deduced amino acid using DNA to protein-translated program (FastPCR) and then both sequences of nucleotide and amino acid were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw). The partial cDNA encoding PAL (467 bp) and LgPOD (464 bp) genes of mangosteen pericarp were identified. After alignment of the partial deduced amino acid sequences with other plants, PAL (GenBank accession number ABU63711.1) gene of mangosteen pericarp shared 92-95% homology with *Populas tremuloides*, *Citrus clementina* x *Citrus reticulate*, *Manihot esculenta*, *Nicotiana attenuate*, *Beta vulgaris* and *Rhizophara mangle*, respectively. The LgPOD (GenBank accession number ABU63712.1) gene of mangosteen pericarp shared 60-62% homology with *Nicotiana tabacum*, *Gossypium hirsutum*, *Ipomoea batatas*, *Populus kitakamiensis* and *Solanum lycopersicum*, respectively. Highly conserved of PAL and LgPOD genes of mangosteen pericarp were shown in Figures 23 and 24.

The partial cDNA encoding 18S rRNA (512 bp) and GAPDH (409 bp) genes were also identified. 18S rRNA (GenBank accession number EU032463) gene of mangosteen (*Garcinia mangostana* L.) pericarp shared 99% homology with *Garcinia subelliptica* (AB233538.1) and GAPDH (GenBank accession number EU032465) gene shared 81% homology with *Capsicum annuum* (data not shown).

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Amer. mangrove  NAGEAFRLAGIESGFFELQPKEGLALVNGTAVGSGLASMVLFEANILAVLAEMLSAIFAE 294
Mangosteen      -------------------------------------------------------VIFAE 5
Cassava         DAQQAFHSAGIDSGFFELQPKEGLALVNGTAVGSGLASMVLFEANVLAVLSEVLSAIFAE 288
Populus         DAVEAFKAAGIDSGFFELQPKEGLALVNGTAVGSGLASMVLFETNVLAVLSELISAIFAE 290
Citrus          DAQEASKQAGFG--FFELQPKEGLALVNGTAVGSGLASMVLFDANNLALLSEILSAIFAE 297
Tobacco         NAEEAFRVAGVNGGFFELQPKEGLALVNGTAVGSGMASMVLFDSNILAVMSEVLSAIFAE 293
Beetroot        NAEQAFKAAQISSGFFELQPKEGLALVNGTAVGSGMASIVLFETNILAVLAEVISAVFAE 295
                                                                       .:***

Amer. mangrove  VMNGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYMKEAKRIHEMDPLQKPKQDRYA 354
Mangosteen      VMNGKAEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYMKEAKRLHEMDPLQKPKQDRYA 65
Cassava         VMNGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYIKAAKKLHEIDPLQKPKQDRYA 348
Populus         VMNGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSAYMKAAKKLHEMDPLQKPKQDRYA 350
Citrus          VMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYVKAAKKLHEIDPLQKPKQDRYA 357
Tobacco         VMNGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYVKAAQKLHEMDPLQKPKQDRYA 353
Beetroot        VMNGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYMKAAKALHELDPLQKPKQDRYA 355
                **:**.******************************:*:* *: :**:************

Amer. mangrove  LRTSPQWLGPQIEVIRFSTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNA 414
Mangosteen      LRTSPQWLGPPIEVIRFATKMIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGMSMDNA 125
Cassava         LRTSPQWLGPQIEVIRFSTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNA 408
Populus         LRTSPQWLGPQIEVIRFSTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNV 410
Citrus          LRTSPQWLGPQIEVIRFATKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNT 417
Tobacco         LRTSPQWLGPQIEVIRSATKMIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNA 413
Beetroot        LRTSPQWLGPQIEVIRFATKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGVSMDNA 415
                ********** ***** :** *********************************:****.

Amer. mangrove  RLAIAAIGKLMFAQFSELVNDFYNNGLPSNLTGGSNPSLDYGFKGAEIAMASYCSELQYL 474
Mangosteen      RLAIAAIGKLMFAQFSELVNDYYNNGLPSN------------------------------ 155
Cassava         RLAIASIGKLMFAQFSELVNDFYNNGLPSNLTASRNPSLDYGFKGAEIAMASYCSELQYL 468
Populus         RLAIASIGKLLFAQFSELVNDFYNNGLPSNLTASRNPSLDYGFKGAEIAMASYCSELQYL 470
Citrus          RLAIAAIGKLMFAQFSELVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCSELQFL 477
Tobacco         RLALASIGKLMFAQFSELVNDYYNNGLPSNLTASRNPSLDYGFKGAEIAMASYCSELQFL 473
Beetroot        RLAIAAIGKLLFAQFSELVNDYYNNGLPSNLTASRDPSLDYGFKGAEIAMASYCSELQFL 475
                ***:*:****:**********:********
```

**Figure 23** Alignment of partial deduced amino acid sequences of mangosteen PAL cDNA fragment with other plants, American mangrove (AAW51923.2), cassava (AAK60275.1), populus (AAN52279.1), citrus (CAB42793.1), tobacco (ABG75911.1) and beetroot (CAH17686.1). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

```
Tomato           RLHFHDCFVNGCDGSLLLDNAAG-IESEKDAASN-VGAGGFDIVDDIKTALENVCPGVVS 117
Tobacco          RLHFHDCFVNGCDGSILLD-TDG-TQTEKDAPAN-VGAGGFDIVDDIKTALENVCPGVVS 116
Populus          RLHFHDCFVNGCDGSILLDNT-DTIESEKEAAPNNNSVRGFDVVDDMKAALENACPGIVS 86
Sweet potato     RLHFHDCFVDGCDGSLLLDNNGTTIVSEKDALPNTNSTRGFDVVDNIKTAVENACPGVVS 120
Mangosteen       --HFHDCFVNGCDGSVLLDGT----DSEQNAQPN-QALGGFSVVDDIKTALETSCPGVVS 53
Gossypium        RLHFHDCFVHGCDASLLLNGT----DGEKTATPN-LSTEGYEVIDDIKTALEKACPRVVS 115
                   *******.***.*:**:        *: * .*  .  *:.::*::*:*:*. ** :**

Tomato           CADILALASEIGVALVGGPTWQVLLGRRDSLTANRSGVDSDIPTPFESLDVMRPQFTNKG 177
Tobacco          CADILALASEIGVVLAKGPSWQVLFGRKDSLTANRSGANSDIPSPFETLAVMIPQFTNKG 176
Populus          CADILAIAAEQSVCLAGGPSWTVPLGRRDSLIANRSGANSALPSPFASLDVLKSKFAAVG 146
Sweet potato     CVDILALASESSVSLAGGPSWNVLLGRRDRRTANQGGANTSLPSPFENLTNLTQKFTNVG 180
Mangosteen       CADILALAAEIMVSMAGGPTWNVQLGRRDGLSANSAGT-TAIPLSSDTLDILTEKFSAKG 112
Gossypium        CADVLALAAQISVSLGGGPKWQVPLGRRDSLTAHREGT-GSIPTGHESLANIATLFKSVG 174
                 *.*:**:*::  * :  **.* * :**:*   *:  *.   :*    .*  :   *   *

Tomato           MDIT-DLVALSGAHTFGRARCGTFQQRLFNFSG-SGSPDPTINSTYLPTLQATCPQGGNN 235
Tobacco          MDLT-DLVALSGAHTFGRARCGTFEQRLFNFNG-SGNPDLTVDATFLQTLQGICPQGGNN 234
Populus          LDTSSDLVALSGAHTFGRAQCSSFNLRLYNFSG-SGNPDPTLNTTYLAELQQLCPQAGN- 204
Sweet potato     LNVN-DLVALSGAHTFGRAQCRTFSPRLFNFSN-TGNPDPTLNTTYLATLQQICPQGGS- 237
Mangosteen       XDTT-DLVALSGAHTFGVARXIAFSNRLYTFNN-TENPDPTINT---------------- 154
Gossypium        LDST-DLVALSGVHTFGRARCAAFMDRLYNFNNITGKTDPTLNATYANTLKQRCPKGGD- 232
                  : . *******.**** *:  :*  **:.*.. : ..* *:::
```

**Figure 24** Alignment of partial deduced amino acid sequences of mangosteen LgPOD cDNA fragment with other plants, tomato (CAA50597.1), tobacco (P11965), populus (BAA06335.1), sweet potato (CAB494692.1), and gossypium (AAQ67366.1). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that conserved substitutions have been observed. The dot (.) means that semi-conserved substitutions are observed.

2.3.1 Northern analysis using digoxygenin-11-UTP (DIG) labeling probes

1) Effect of low temperature on pericarp hardening of mangosteen fruit

Total RNA was isolated from pericarp of reddish purple mangosteen fruit stored at 6 and 12°C for 12 days. Northern blot analysis revealed that PAL mRNA transcription was induced by low temperature storage, increasing with storage time until day 9, and then decreasing thereafter. Fruit stored at 6°C had greater PAL mRNA accumulation than that stored at 12°C (Figure 25A). LgPOD mRNA accumulation in fruit stored at 6°C increased to a maximum on day 9, and then decreased thereafter, although there was variability across the first 9 days of storage. Expression at 12°C did not change much with storage time (Figure 25B).

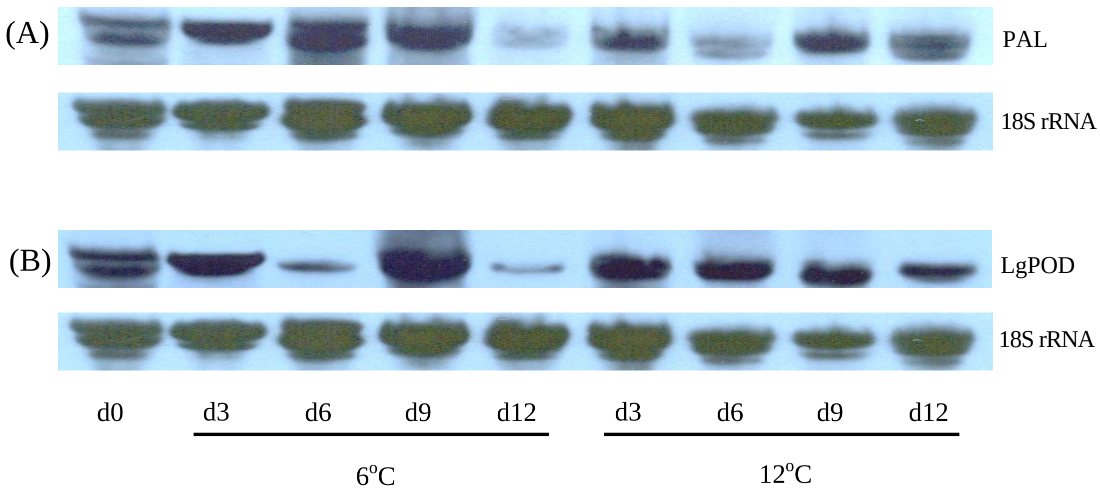


**Figure 25** Northern hybridization analysis of (A) PAL and (B) LgPOD expression in reddish purple mangosteen fruit stored at 6 and 12°C for 12 days. Equal loading of RNA was checked by hybridization with a mangosteen 18S rRNA probe.

2) Effect of low $O_2$ applied during low temperature storage on pericarp hardening

Total RNA was isolated from pericarp of reddish purple mangosteen fruit stored at 6°C in normal air and low $O_2$, and then transferred to room temperature for 3 days. PAL mRNA accumulation increased when the fruit were exposed to low temperature and then slightly decreased. Fruit stored at 6°C in normal air had greater PAL mRNA accumulation than those stored in low $O_2$. Upon transfer to room temperature, PAL mRNA transcripts increased and fruit stored in normal air showed greater PAL mRNA transcripts than those stored in low $O_2$ (Figure 26A). LgPOD mRNA accumulation of fruit stored in normal air increased continuously and greater than those stored in low $O_2$. Upon transfer to room temperature, LgPOD mRNA transcripts of fruit stored in normal air increased at day 6 and decreased thereafter, whereas LgPOD mRNA in low $O_2$ increased sharply on day 3 and then decreased thereafter (Figure 26B).

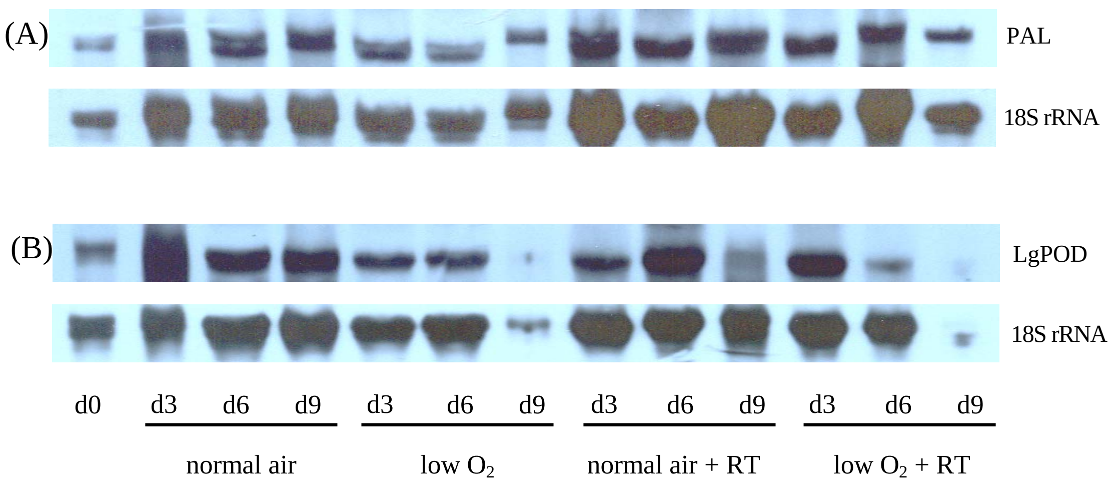


**Figure 26** Northern hybridization analysis of (A) PAL and (B) LgPOD expression in reddish purple mangosteen fruit stored at 6°C in normal air and low $O_2$ conditions and measured immediately and after transfer to room temperature for 3 days. Equal loading of RNA was checked by hybridization with a mangosteen 18S rRNA probe.

3) Effect of low $O_2$ applied after low temperature storage on pericarp hardening

Total RNA was isolated from pericarp of reddish purple mangosteen fruit stored at 6$^{o}$C and transferred to room temperature for 3 days in normal air and low $O_2$. The level of PAL mRNA transcripts decreased with time. Upon transfer to room temperature, PAL transcripts of fruit stored in normal air were maintained, but were barely detectable in low $O_2$ (Figure 27A). The level of LgPOD mRNA transcripts increased with time after transfer the fruit from 6$^{o}$C to room temperature. LgPOD mRNA transcripts of fruit stored in normal air was greater than those stored in low $O_2$ (Figure 27B).

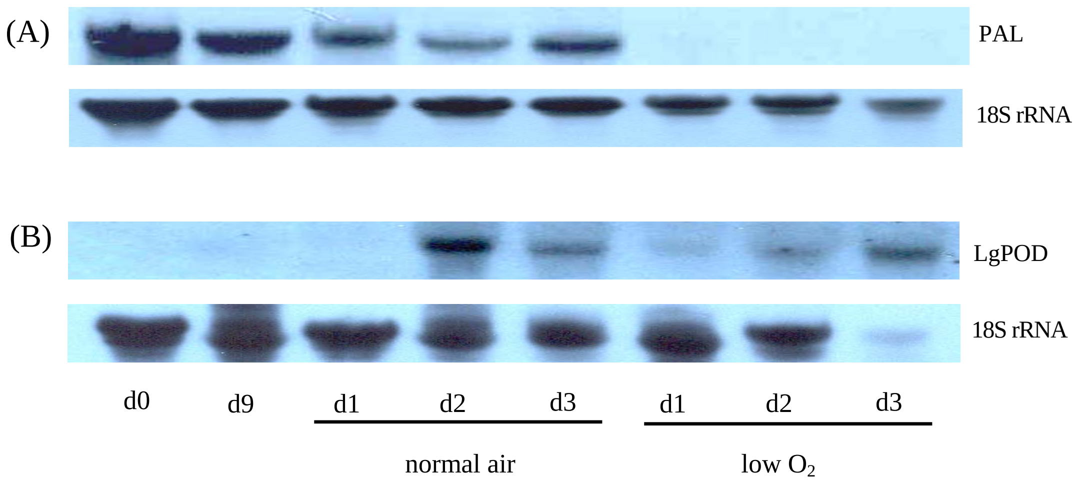


**Figure 27** Northern hybridization analysis of (A) PAL and (B) LgPOD expression in reddish purple mangosteen fruit stored at 6$^{o}$C for 9 days and transferred to room temperature for 3 days in normal air and low $O_2$ conditions. Equal loading of RNA was checked by hybridization with a mangosteen 18S rRNA probe.

2.3.2 Semi–quantitative RT-PCR

1) Effect of low temperature on pericarp hardening of mangosteen fruit

PAL mRNA accumulation in reddish purple fruit stored at 6°C increased to a maximum on day 6 and then decreased thereafter, whereas fruit stored at 12°C increased to a maximum on day 9 and then decreased thereafter. LgPOD mRNA accumulation in fruit stored at 6°C increased to a maximum on day 6 and then decreased thereafter. Fruit stored at 12°C, LgPOD mRNA accumulation increased to a maximum on day 9 and day 12 (Figure 28).

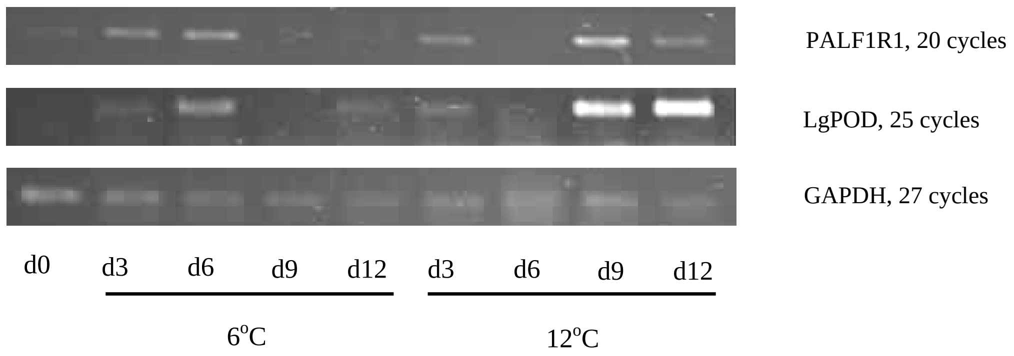


**Figure 28** Semi-quantitative RT-PCR of PAL and LgPOD expression in reddish purple mangosteen fruit stored at 6 and 12°C for 12 days.

2) Effect of low $O_2$ applied during low temperature storage on pericarp hardening

PAL mRNA accumulation in reddish purple fruit stored at 6°C in normal air increased sharply at 3 days and then decreased thereafter, although transcripts were still at higher levels than on the first day (day 0). In 6°C storage, PAL mRNA accumulation of fruit stored in normal air was slightly greater than in fruit stored in low $O_2$. PAL mRNA in fruit stored in both conditions did not change much after transfer to room temperature, and accumulation in fruit stored in normal air was greater than in fruit stored in low $O_2$ (Figure 29). The results of semi-quantitative RT-PCR with another PAL primer set (MgstpalF1 and MgstpalR1) provided similar results of mRNA accumulation (data not shown).

At the 3 first days in normal air, LgPOD mRNA accumulation increased sharply and then decreased, while accumulation in low $O_2$ was low. After transfer to room temperature, LgPOD mRNA accumulation in normal air increased especially at the end of storage, while LgPOD mRNA accumulation in low $O_2$ was barely detectable (Figure 29).

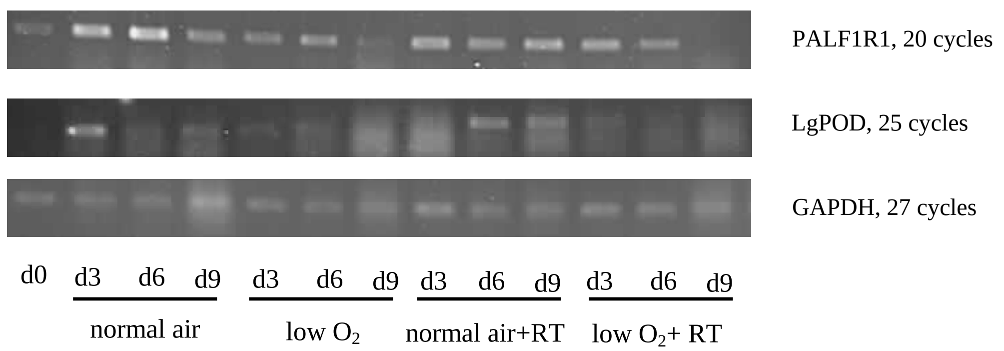


**Figure 29** Semi-quantitative RT-PCR of PAL and LgPOD expression in reddish purple mangosteen fruit stored at 6°C in normal air and low $O_2$ conditions and measured immediately and after transfer to room temperature for 3 days.

3) Effect of low $O_2$ applied after low temperature storage on pericarp hardening

PAL mRNA accumulation in fruit stored at 6°C for 9 days increased. After transfer to room temperature, PAL mRNA accumulation decreased immediately, but increased up to 3 days. When comparing the storage conditions, accumulation in normal air was greater than in low $O_2$.

LgPOD mRNA accumulation was low in 6°C storage, but accumulation increased after transfer to room temperature. LgPOD mRNA accumulation in normal air was greater than in low $O_2$ (Figure 30).

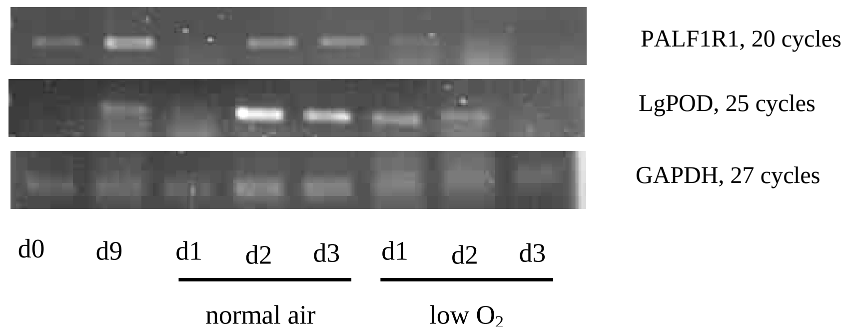


**Figure 30** Semi-quantitative RT-PCR of PAL and LgPOD expression in reddish purple mangosteen fruit stored at 6°C for 9 days and transferred to room temperature for 3 days in normal air and low $O_2$ conditions.

### 2.3.3 Real-time PCR

1) Effect of low temperature on the pericarp hardening of mangosteen fruit

Relative expression of the PAL gene in reddish purple fruit stored at 6$^{o}$C increased continuously to a maximum at day 9 and then decreased thereafter, whereas fruit stored at 12$^{o}$C increased to a maximum at day 6 and then decreased thereafter (Figure 31).

Relative expression of LgPOD gene in reddish purple fruit stored at 6$^{o}$C increased to a maximum at first 3 days and then decreased thereafter, whereas relative expression in fruit stored at 12$^{o}$C fluctuated, but expression was higher than fruit stored at 6$^{o}$C (Figure 32).

2) Effect of low $O_2$ applied during low temperature storage on pericarp hardening

Relative expression of the PAL gene in reddish purple fruit stored at 6$^{o}$C in normal air increased slightly to a maximum at first 3 days and decreased thereafter, whereas relative expression in low $O_2$ increased throughout the storage time. After transfer to room temperature, relative expression of PAL gene in fruit stored in normal air increased to maximum on day 6 and then decreased thereafter, but increased to a maximum at first 3 days and then decreased in low $O_2$ storage (Figure 33).

Relative expression of LgPOD gene in reddish purple fruit stored at 6$^{o}$C in normal air did not change, whereas relative expression of LgPOD gene in low $O_2$ increased to a maximum on day 6 and decreased thereafter. After transfer to room temperature, relative expression of LgPOD gene in normal air and low $O_2$ increased slightly at the end of storage (Figure 34).

3) Effect of low $O_2$ applied after low temperature storage on pericarp hardening

Relative expression of the PAL gene in reddish purple fruit stored at 6$^{o}$C was stable. After transfer to room temperature, expression of PAL gene in fruit stored in normal air increased continuously to a maximum on day 2 and then decreased. At the end of storage in room temperature, expression was higher than on the first day. Expression in fruit stored in low $O_2$ increased continuously, and was higher than fruit stored in normal air (Figure 35).

Relative expression of LgPOD gene in reddish purple fruit stored at 6$^{o}$C for 9 days increased. After transfer to room temperature, expression in fruit stored in normal air increased continuously to a maximum on day 2 and then decreased and was still higher than on the first day. Expression in fruit stored in low $O_2$ increased sharply only at the end of storage (Figure 36).

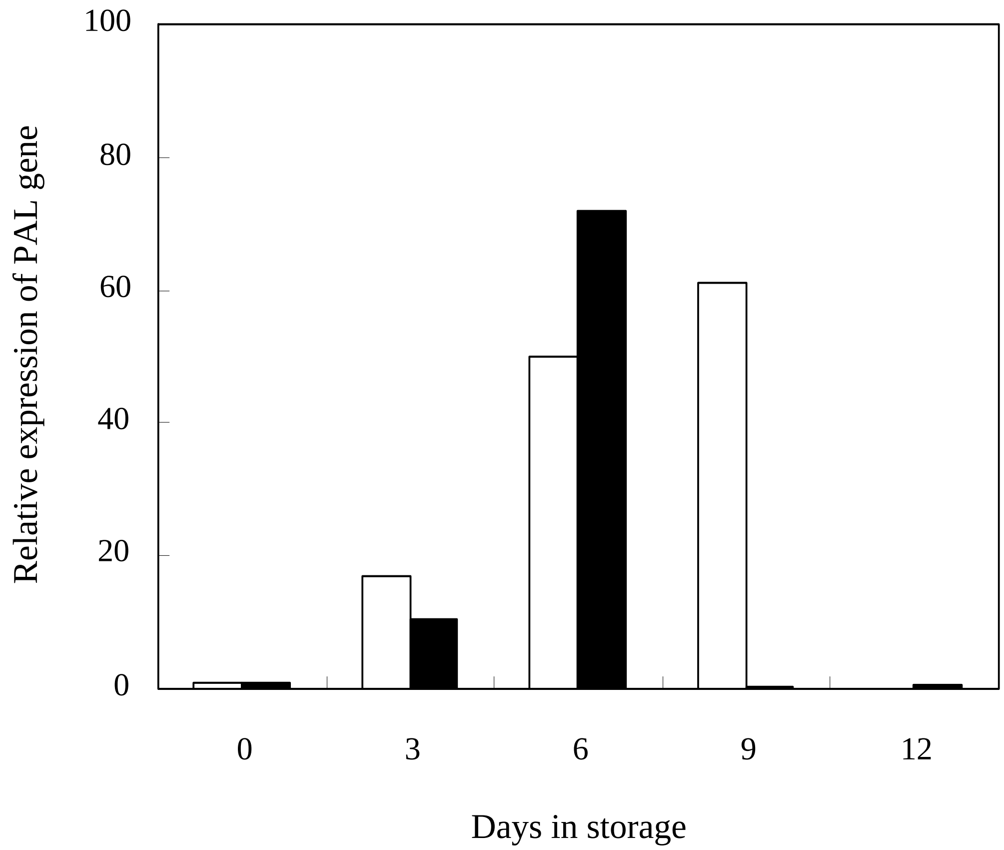


**Figure 31** Relative expression of PAL gene in reddish purple mangosteen fruit stored at 6°C (□) and 12°C (■) for 12 days.

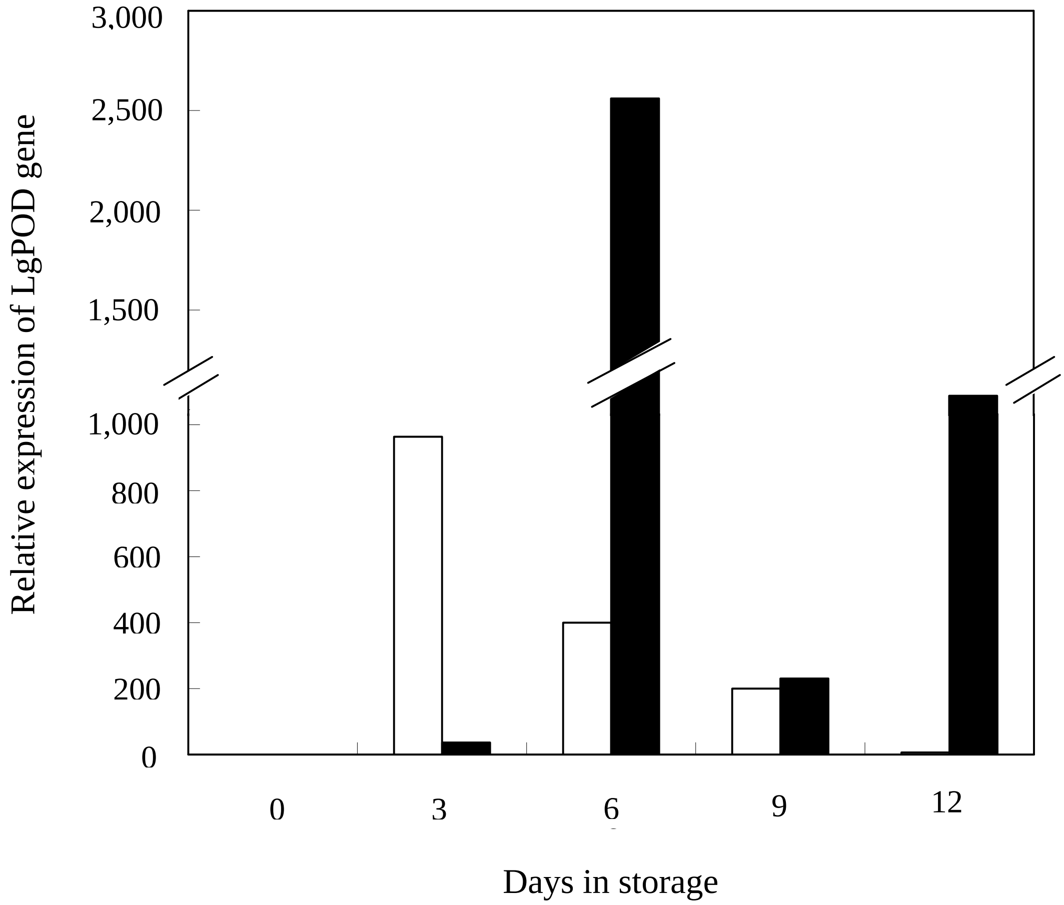


**Figure 32** Relative expression of LgPOD gene in reddish purple mangosteen fruit stored at 6$^{o}$C (□) and 12$^{o}$C (■) for 12 days.

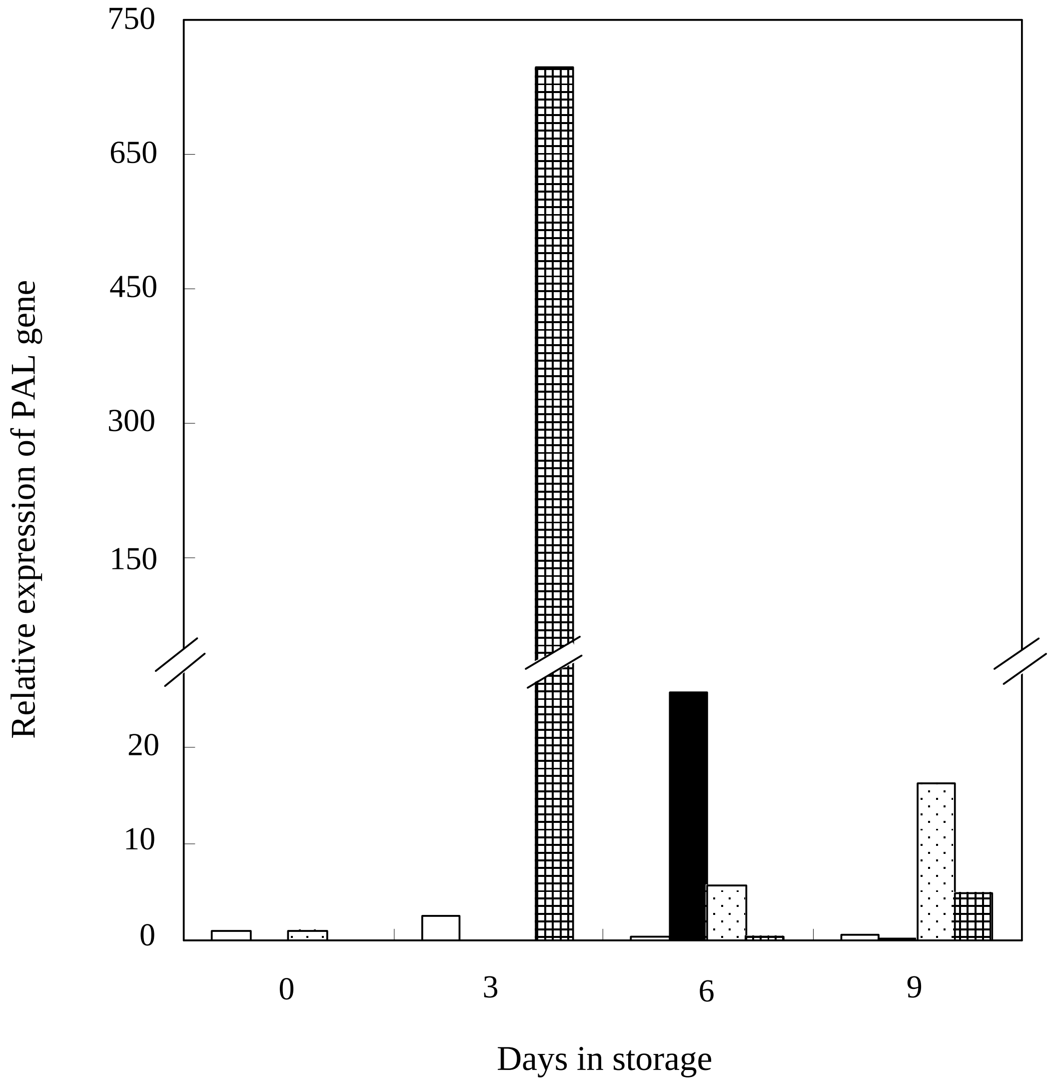


**Figure 33** Relative expression of PAL gene in reddish purple mangosteen fruit stored at 6°C in normal air (□,■) and low $O_2$ (◘,▦) conditions and measured immediately (□,◘) and after transfer to room temperature for 3 days (■,▦).

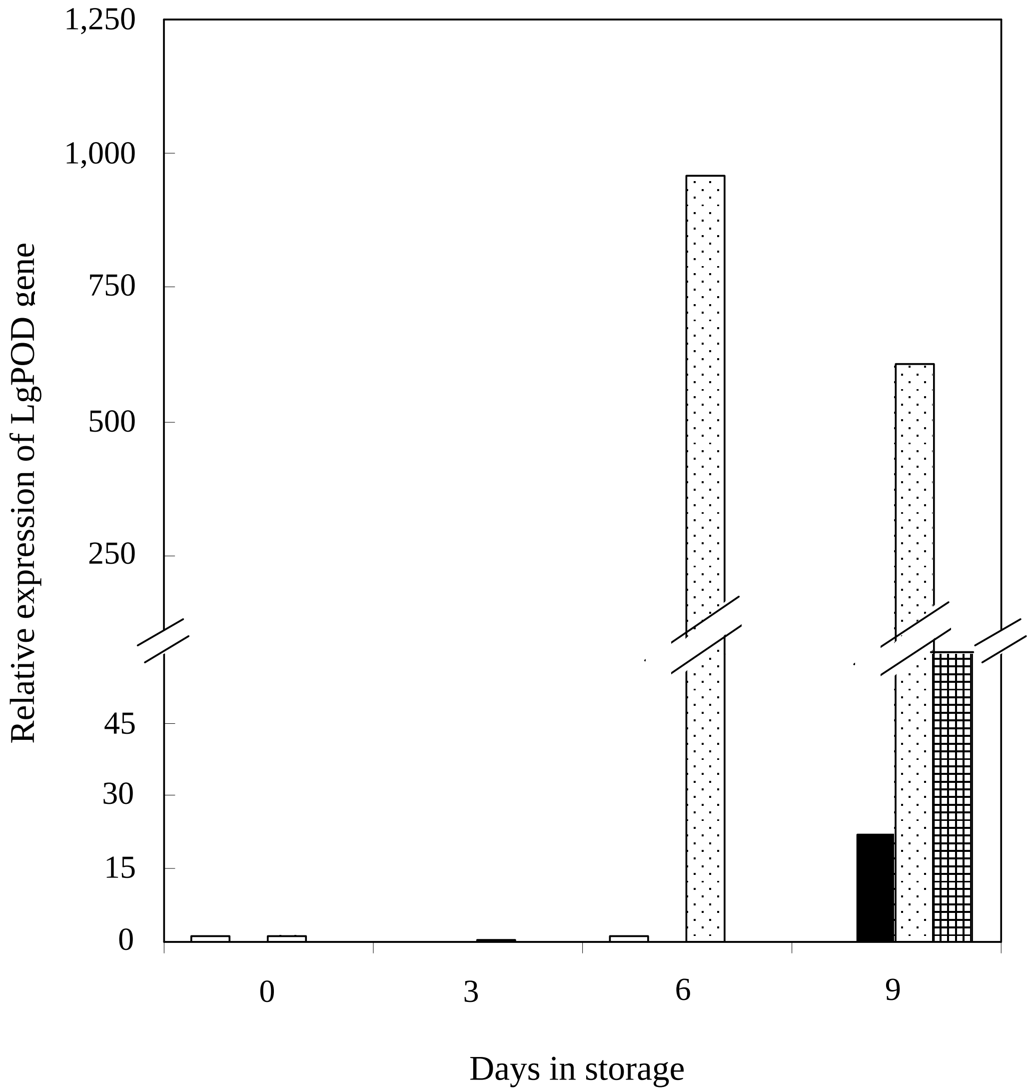


**Figure 34** Relative expression of LgPOD gene in reddish purple mangosteen fruit stored at 6$^{o}$C in normal air (□,■) and low $O_2$ (⊡,▦) conditions and measured immediately (□,⊡) and after transfer to room temperature for 3 days (■,▦).

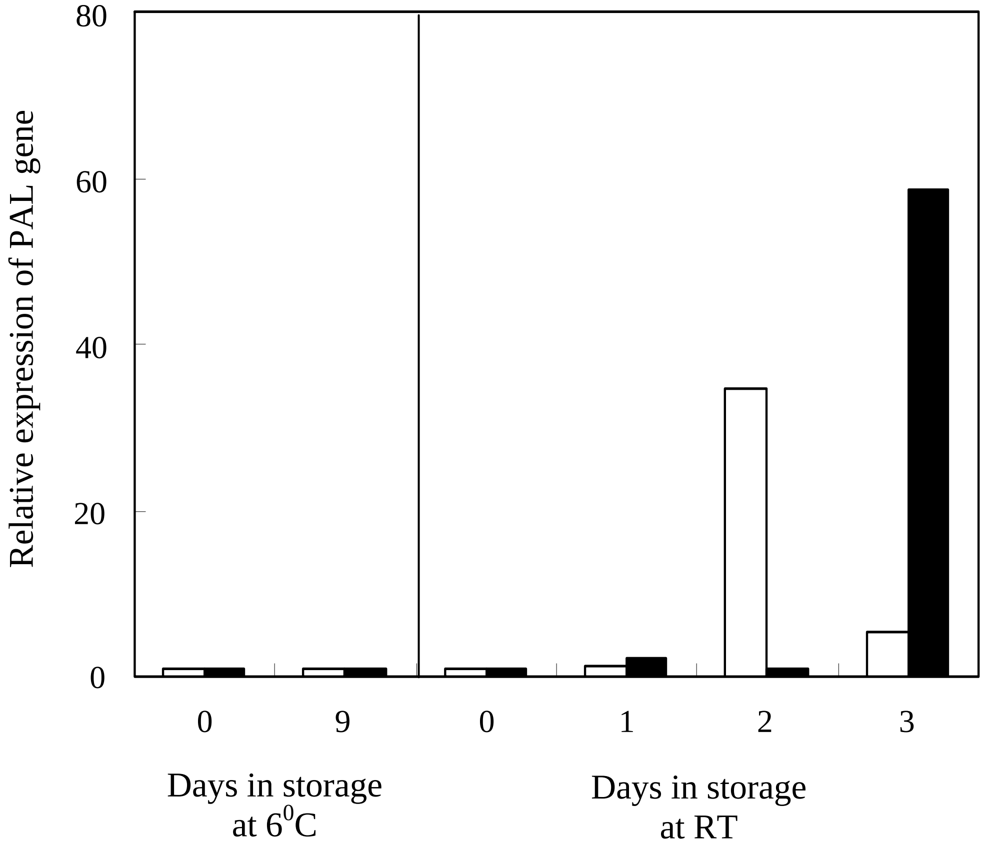


**Figure 35** Relative expression of PAL gene in reddish purple mangosteen fruit stored at 6$^o$C for 9 days and transferred to room temperature for 3 days in normal air (□) and low $O_2$ (■) conditions.

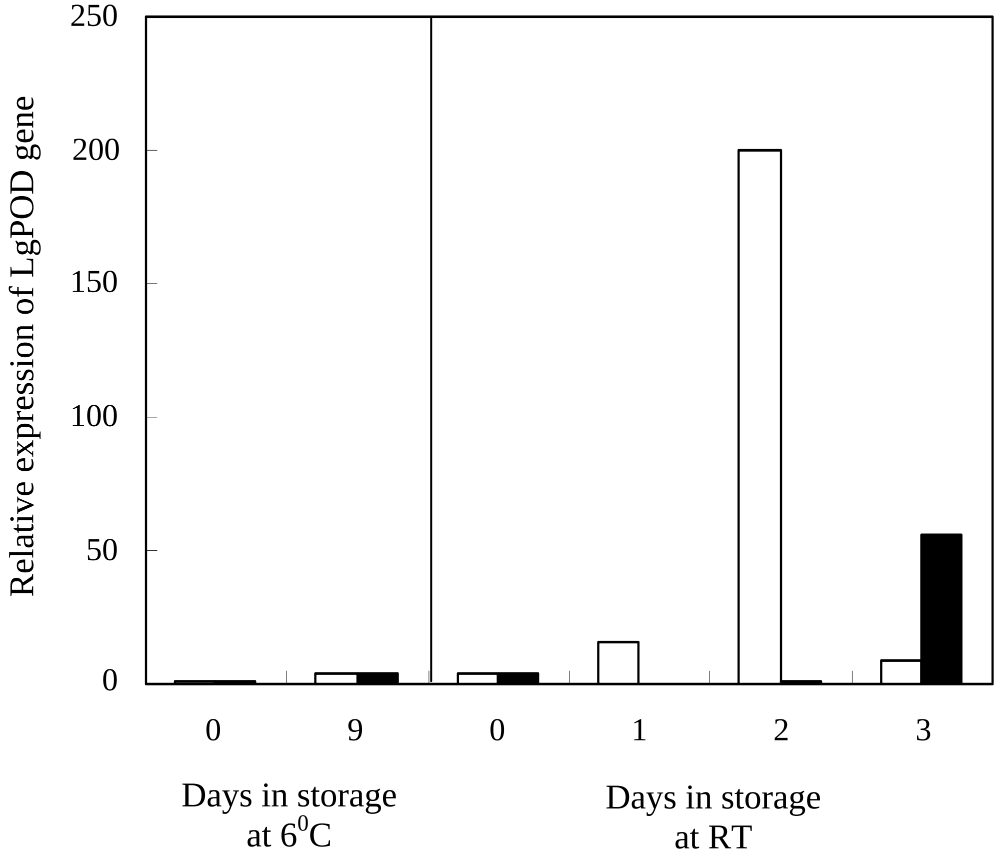


**Figure 36** Relative expression of LgPOD gene in reddish purple mangosteen fruit stored at 6$^{o}$C for 9 days and transferred to room temperature for 3 days in normal air (□) and low $O_2$ (■) conditions.