

Article

Determination of optimal harvest time for preventing seed browning associated with hot pepper seed maturity during cold storage

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Abstract: Chilling injury (CI), which causes seed browning in pepper, may arise following long-term cold storage, and is a major cause of postharvest losses. To explore potential strategies of minimizing the associated postharvest losses, the present study investigated the optimal pepper harvest time that could reduce levels of seed browning, in addition to the relationship between fruit maturity and seed browning. Fruit harvested 15 days after flowering (DAF) were sensitive to cold storage at 4°C and exhibited 100% seed browning (CI index, 4.0); in contrast, the seed browning rate of fruit harvested 35 DAF was 10% (CI index, 0.4) within 7 d of cold storage. Seed antioxidant activity was higher in seeds harvested at early stages (15 DAF to 20 DAF) than in seeds harvested at later stages (40 DAF to 50 DAF) at the beginning of storage. Pericarps of fruit harvested at 50 DAF exhibited the highest antioxidant activity. Lipoygenase, catalase, and peroxidase activity, and the expression levels of cell wall-related genes, pectin methylesterase-like protein, and endo- β -1,4-glucanase were higher in seeds of immature fruit harvested 15 DAF than in seeds of mature fruit harvested 35 DAF. The endosperm separated from the seed coat in fruit harvested 35 DAF and the seeds did not brown under low-temperature storage. The lack of seed browning observed in mature fruit under low-temperature storage could be attributed to physical protection provided by the seed coat rather than cold stress resistance conferred by antioxidants.

Keywords: chilling injury, pepper, seed browning, maturity, harvest time

1 Introduction

Hot pepper (*Capsicum annuum* L.) is a subtropical crop that is susceptible to chilling injury (CI). CI is observed in hot pepper subjected to < 7–10°C for 1–2 weeks [1,2]. The symptoms of CI in pepper include seed browning, surface pitting, shrinkage, and calyx discoloration [2-4]. However, chilling injury severity could vary across cultivars, fruit maturity levels, and exposure times [5]. Postharvest CI decreases pepper fruit quality, which causes yield losses during cold storage and in the course of market distribution [6-8]. Nevertheless, cold storage is an effective postharvest management technology for the maintenance of horticultural crop quality and the suppression of respiration [2]; consequently, it is critical to investigate appropriate strategies of storing and maintaining the quality of such sensitive and commercially important crops.

Seed browning is major symptom of CI in hot pepper [9]. Severe seed browning has been observed in hot pepper stored at 5°C, which has been attributed to invisible cell damage and the release of free phenolic compounds [9,10]. Under severe CI, the exocarp turns brown or black, and the epidermis and hypodermal cortical cells undergo necrosis [11,12]. Browning symptoms could be minimized by reducing storage and distribution periods. In previous studies exploring mechanisms of reducing seed browning, seed browning was significantly inhibited by Methyl jasmonate (MeJA) but induced by Methyl Salicylate during storage at 2°C; in addition, treatment with MeJA inhibited seed browning by increasing endogenous JA production and upregulating the expression of antioxidant-related genes in pepper fruit [2,10].

It has been proposed that CI induces oxidative stress responses and that antioxidants protect the fruit against cold-induced damage [13]. Similarly, CI symptoms have been attributed to cell membrane damage by reactive oxygen species (ROS) generated from lipid peroxidation [5,6]. CI increases membrane permeability and the leakage of ions from the cytoplasm into the intercellular spaces [14], and results in ultrastructural changes to the membrane [15].

However, CI sensitivity also depends on crop maturity. Mature persimmon and tomato fruit were relatively less sensitive to CI than immature fruit [16]. In addition, green bell pepper was more sensitive to surface pitting than mature pepper fruit [17]. Chilling stress responses in the form of seed browning have also been observed in young eggplant fruit, with young seeds being more susceptible to chilling-induced browning than old seeds [5,18]. The determination of optimal harvest time of subtropical crops such as pepper could be an effective method of reducing postharvest losses associated with CI; however, few studies have examined the correlation between harvest time, based on fruit maturity, and seed browning under cold storage. Most previous studies have only explored the physiological changes that occur in pepper fruit exposed to CI [19], the metabolite changes in brown seeds under various storage temperatures [20], and seed browning based on different early harvest times following low-temperature storage [5].

Here, to explore a strategy for reducing postharvest losses and maintain hot pepper quality during cold storage and in the course of market distribution, we investigated the optimal harvest time for preventing seed browning induced by CI. We examined changes in fruit quality, antioxidant activity, gene expression, and anatomy at different stages of fruit maturity, namely, 15, 20, 25, 30, 35, 40, 45, and 50 days after flowering (DAF), following cold storage. The results of the present study could not only enhance our understanding of the mechanism of seed browning following CI but also facilitate the formulation of effective postharvest management approaches.

2 Materials and Methods

2.1 Plant materials and storage conditions

Pepper (*Capsicum annuum* L. “Nockgwang”) plants were grown in the greenhouses of the National Institute of Horticultural and Herbal Science, Wanju, Republic of Korea. The fruits were harvested at 15, 20, 25, 30, 35, 40, 45, and 50 DAF (Figure 1A). Harvested fruit were washed with water containing 200 µL L⁻¹ sodium hypochlorite and air-dried at room temperature (25–28°C). They were then either packed in a commercial cardboard box covered with plastic film and stored at 4°C for 14 d or maintained at 20°C for 8 d. The relative humidity was maintained at 85 ± 5%. Fruits were evaluated following the storage. Seeds and pericarp were excised for antioxidant activity analyses and RNA extraction, flash-frozen in liquid nitrogen, and stored at -80°C.

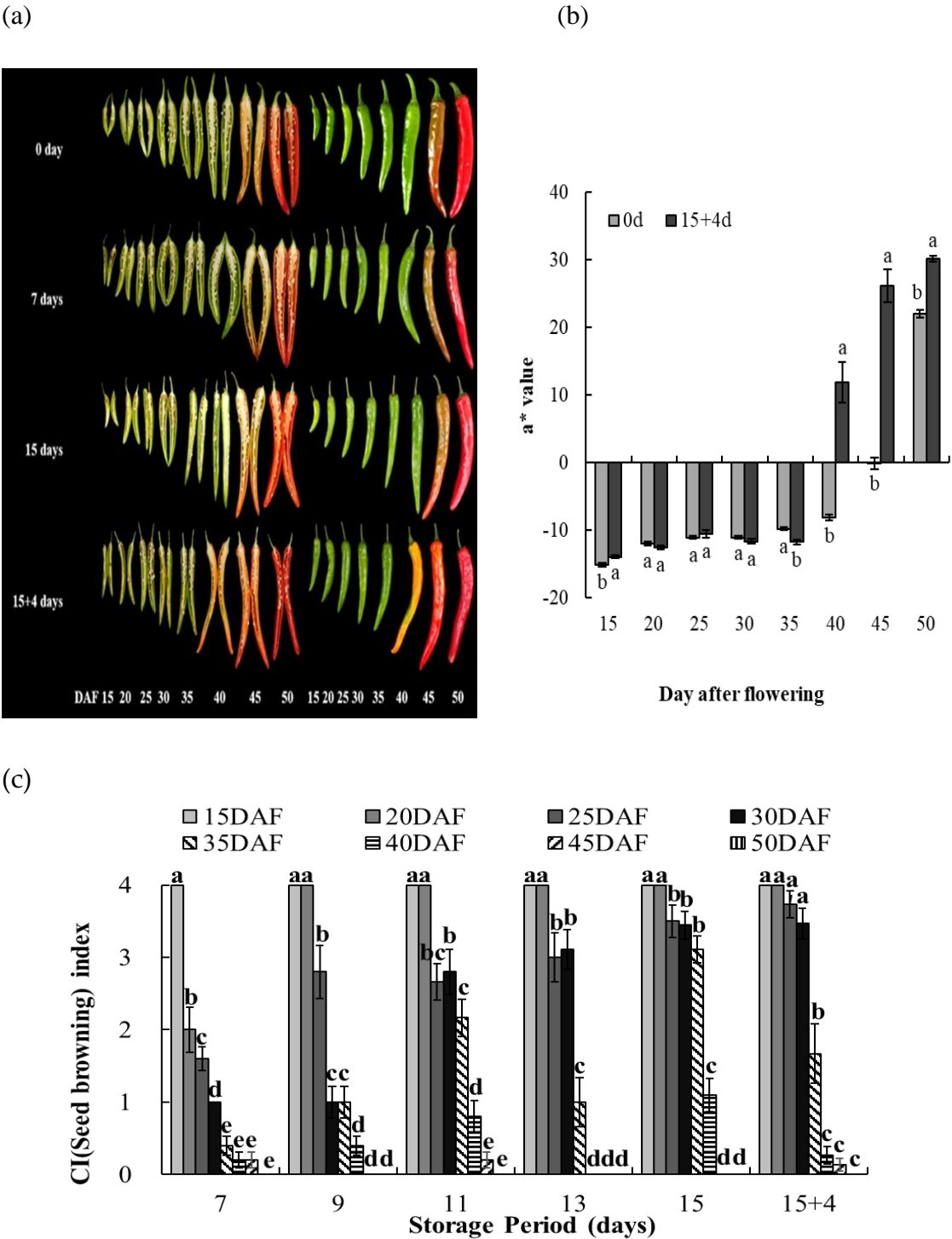


Figure 1. Changes in pepper fruit skin color and seed browning during storage. (A) Photograph of seeds and pericarp of pepper fruit. (B) Changes in skin color a^* value (redness) in fruit harvested at different times 0 d and 15 d after cold storage and maintained at 20°C for 4 d (15+4d). (C) CI (Seed browning) index. Data are means \pm standard error (SE) ($n = 20$). Different letters indicate significant difference among treatments at the same storage time according to the Duncan's New Multiple Range (DMRT) test. $P < 0.05$. Fruits were harvested at 15, 20, 25, 30, 35, 40, 45, and 50 days after flowering (DAF), stored for 15 d at 4 °C and for 4 d at 20 °C (15+4).

2.2 Fruit quality evaluation

For the firmness assay, 20 pepper fruits were selected from the different harvest time treatments and two portions of each fruit were measured with a texture analyzer (TA Plus Lloyd Instruments Ltd., Fareham, Hampshire, UK) fitted with a plunger head 3 mm in diameter. Its operating speed was 0.5 mm s⁻¹. Fifteen fruit samples were used to measure total soluble solids (TSS) content and titratable acidity (TA) with a digital refractometer (PAL-1; Atago Co. Ltd., Saitama, Japan), and an auto pH titrator (TitroLine Easy; Schott Instruments GmbH, Mainz, Germany). TA was expressed in g acetic acid/100 g sample juice. For the fruit skin color assessment, 15 samples per treatment were measured and two portions of each fruit were examined using a color difference meter (Model CR-400, Minolta, Osaka, Japan) based on Hunter's a* scale.

The CI index was calculated according to the degree of seed browning, which was visually rated as follows: 0 = no symptom; 1 = < 25%; 2 = 25–50%; 3 = 50–75%; 4+ = > 75%. The CI index was calculated based on the following formula [21]:

$$\text{CI (Seed browning) index} = [\Sigma (\text{chilling injury grade}) \times (\text{number of fruit at the chilling injury grade})] / (\text{total number of fruits in the treatment}) \quad (1)$$

2.3 Antioxidant, total phenol analysis

Each sample was freeze-dried and pulverized. The seed powder (100 mg) or pericarp powder (50 mg) was extracted twice in 1 mL of 70% (v/v) ethanol with shaking for 1 h at 20 ± 2°C. The extracts were centrifuged at 4°C at 12,000 × g for 15 min. The supernatant was passed through a 0.2-µm Teflon® (PTFE) filter and used in the antioxidant activity assay.

The total polyphenol content was measured according to the method of Singleton and Rossi [22], with modifications. Briefly, 100 µL extract or standard was mixed with 100 µL of 1 N Folin–Ciocalteu reagent for 3 min and then 1 mL of 2% (w/v) sodium carbonate solution was added. The mixture was incubated for 30 min and its absorbance was read at 726 nm using a microplate reader (EPOCH2, BioTek Instruments Inc. VT, USA). The output was expressed as mM gallic acid equivalents (GAE)/g DW.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was assessed according to the method of Dietz et al. [23]. Twenty microliters of extract was mixed with 180 µL of 0.18 mM DPPH and briefly vortexed. The samples were set aside for 20 min and the absorbance was measured at 515 nm using a microplate reader. DPPH-free methanol served as the blank reference. The absorbance was converted to DPPH radical scavenging activity. 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was evaluated according to the method of Re et al. [24], with modifications. In brief, 7 mM ABTS stock solution was prepared by combining 7 mM ABTS with 2.45 mM potassium persulfate and the mixture was stored in the dark for 16 h. The solution was adjusted with ethanol until the absorbance at 734 nm was 0.7 ± 0.02 at ambient temperature (25 ± 2°C). Subsequently, 20 µL of each extract was mixed with 180 µL of the ABTS+ solution and the absorbance at 734 nm was measured using a microplate reader at ambient temperature (25 ± 2°C) after 10 min. The ABTS radical scavenging activity was expressed as Trolox equivalent antioxidant capacity (µmol TE/g DW).

2.4 Gene expression analysis

All samples were frozen in liquid nitrogen and pulverized to a fine powder in a mortar and pestle. Total RNA was extracted from the pericarp tissue using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA). Five hundred nanograms of total RNA was used to synthesize cDNA in a QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA). The product was amplified using iQ™ SYBR Green Supermix and specific primers (Supplementary Table S1). RT-qPCR was performed in a CFX96 Touch™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The PCR was programmed as

follows: 95 °C for 30 s, followed by 40 cycles of 95°C for 10 s, and 55 °C or 58 °C for 40 s. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen [25] and normalized against the expression levels of the housekeeping genes, *Actin* and eukaryotic initiation factor 4E (*eIF4E*). The qRT-PCR analysis was conducted using at least 3 and 2 biological and technical replicates, respectively.

2.5 Anatomical analysis under light microscopy and scanning electron microscopy (SEM)

Stereomicroscopy (SM): Live seeds collected at each harvest time were observed under a helium stereomicroscope (Stemi-2000; Carl Zeiss AG, Oberkochen, Germany).

Light microscopy (LM): The procedures used were modified from Clement et al. [26]. Seeds were fixed for 24 h in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) with 4% (w/v) sucrose. After three 30-min rinses in phosphate buffer, the specimens were post-fixed for 4 h with 1% (w/v) OsO_4 in the buffer plus 4% (w/v) sucrose. The samples were then rinsed thrice with buffer for 30 min each time, dehydrated in an alcohol gradient series, immersed in propylene oxide, and embedded in Epon epoxy resin. Semi-thin sections (2.5 μm) were prepared using a ultramicrotome, mounted on glass microscope slides, and subjected to periodic acid-Schiff (PAS) polysaccharide-specific treatment. PAS-positive reactions were red in color. The thin sections were immersed in 1% (w/v) periodic acid for 30 min, and then in Schiff's reagent for 40 min, and finally in 5% (w/v) sodium bisulfite for 35 min. The sections were then rinsed in distilled water, dried on a hotplate, and mounted in Histomount. A negative control was prepared in the same manner, except the periodic acid oxidation step was omitted. The thin sections were observed under a light microscope (Axioscop 2; Carl Zeiss AG, Oberkochen, Germany).

Scanning electron microscopy (SEM): Live tissues were examined under a SEM (SU-3500; Hitachi, Tokyo, Japan) in low vacuum mode.

2.6 Statistical Analysis

Data are means \pm SE. They were subjected to ANOVA and significant differences were evaluated using Duncan's new multiple range test. All data processing was performed in SAS v. 9.2 (SAS Institute, Cary, NC, USA).

3 Results and Discussion

3.1 Fruit quality

The quality of the pepper fruit harvested at different times was determined during low-temperature storage. Fruit firmness increased with a delay in harvest time and decreased with an increase in storage period (Table 1). Firmness of the fruit harvested at 35–45 DAF was significantly higher than in fruit harvested at 15–30 DAF during cold storage at 4°C for 15 d even after transfer to 20°C. Soluble solid content (SSC) significantly increased with an increase in storage period. Similarly, TSS content in bell pepper increased gradually with storage periods [27]. Maximum SSC was observed in the fruit harvested at 50 DAF. The titratable acidity (TA) was higher in the fruit harvested at 35–50 DAF than in fruit harvested earlier. In general, TA reduction is a major characteristic feature during storage periods [28]. Consistently, pepper fruit acidity increased with harvest time showing fruit harvested at 50 DAF had the highest TA while fruit acidity decreased over the storage periods. The fruit harvested at a later stage (35 DAF to 50 DAF) exhibited higher firmness, SSC, and TA than those harvested at 15 DAF. Fruit hardness and flavor improve with post-flowering maturation. There were no significant differences in the color A value (redness) among fruits harvested before 35 DAF. Fruit color changed to red when fruits were transferred to shelf condition at 20°C, after low-temperature storage. Hence, 35 DAF may be the optimal harvest time required to obtain the fruit color and robustness preferred by consumers.

Table 1. Changes in pepper fruit firmness, soluble solid content (SSC), and titratable acidity (TA) during storage.

	0 d	7 d	15 d	15 + 4 d
Firmness (N)				
15 DAF	9.63 ± 0.34 Fa	10.46 ± 0.32 Da	6.51 ± 0.43 CDb	5.12 ± 0.24 CDc
20 DAF	11.05 ± 0.37 Ea	10.63 ± 0.25 Da	6.08 ± 0.26 Db	4.85 ± 0.20 Dc
25 DAF	12.94 ± 0.33 Da	12.62 ± 0.39 Ca	7.28 ± 0.24 Cb	5.35 ± 0.26 CDc
30 DAF	13.80 ± 0.31 CDa	11.82 ± 0.33 Cb	8.67 ± 0.37 Bc	5.15 ± 0.28 CDd
35 DAF	15.76 ± 0.40 Ba	11.94 ± 0.43 Cb	9.23 ± 0.28 Bc	6.55 ± 0.25 Bd
40 DAF	18.91 ± 0.49 Aa	14.42 ± 0.49 Bb	10.71 ± 0.21 Ac	7.55 ± 0.34 Ad
45 DAF	18.16 ± 0.43 Aa	15.97 ± 0.44 Ab	11.20 ± 0.25 Ac	7.94 ± 0.38 Ad
50 DAF	14.75 ± 0.33 BCa	13.97 ± 0.30 Bb	8.65 ± 0.25 Bc	5.73 ± 0.20 Cd
Soluble solids content (%)				
15 DAF	5.53 ± 0.12 Dd	6.40 ± 0.12 DEc	6.90 ± 0.00 Cb	7.70 ± 0.00 Da
20 DAF	5.30 ± 0.06 Ec	6.67 ± 0.03 Dab	6.60 ± 0.00 Cb	6.83 ± 0.09 Ea
25 DAF	5.30 ± 0.00 Ed	6.07 ± 0.13 EFc	6.60 ± 0.06 Cb	7.17 ± 0.15 DEa
30 DAF	5.33 ± 0.03 Ed	5.93 ± 0.03 Fc	6.60 ± 0.06 Cb	7.07 ± 0.15 DEa
35 DAF	5.37 ± 0.03 DEc	6.20 ± 0.17 Efb	6.50 ± 0.06 Cb	6.97 ± 0.18 Ea
40 DAF	5.90 ± 0.00 Cc	7.20 ± 0.12 Cb	6.87 ± 0.15 Cb	8.87 ± 0.50 Ca
45 DAF	7.33 ± 0.07 Bc	8.53 ± 0.15 Bb	9.60 ± 0.12 Ba	9.80 ± 0.25 Ba
50 DAF	9.83 ± 0.03 Ad	10.60 ± 0.17 Ac	11.97 ± 0.32 Ab	13.50 ± 0.00 Aa
Titratable acidity (%)				
15 DAF	0.12 ± 0.00 Ga	0.07 ± 0.00 Fb	0.07 ± 0.00 Gb	0.07 ± 0.00 Db
20 DAF	0.13 ± 0.00 Gb	0.17 ± 0.01 Ea	0.08 ± 0.00 Fc	0.07 ± 0.00 Dc
25 DAF	0.21 ± 0.00 Fa	0.21 ± 0.01 Da	0.10 ± 0.00 DEb	0.10 ± 0.02 Cb
30 DAF	0.25 ± 0.02 Ea	0.23 ± 0.01 Da	0.09 ± 0.00 Eb	0.12 ± 0.02 Cb
35 DAF	0.29 ± 0.01 Da	0.25 ± 0.00 Db	0.10 ± 0.00 Dc	0.11 ± 0.01 Cc
40 DAF	0.34 ± 0.00 Ca	0.34 ± 0.01 Ca	0.11 ± 0.00 Cc	0.20 ± 0.01 Bb
45 DAF	0.45 ± 0.01 Ba	0.45 ± 0.01 Ba	0.19 ± 0.00 Bc	0.23 ± 0.00 ABb
50 DAF	0.52 ± 0.03 Aa	0.54 ± 0.01 Aa	0.22 ± 0.00 Ab	0.24 ± 0.00 Ab

*Fruits were harvested at 15, 20, 25, 30, 35, 40, 45, and 50 days after flowering (DAF) and stored for 15 d at 4 °C (15 d) followed by 4 d at 20 °C (15 + 4 d). Data are means ± SE (n = 20 for firmness; n = 15 for SSC and TA). Means with the same uppercase letter in a column or same lowercase letter in a row are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

3.2 Harvest time and seed browning

Peppers were harvested at 15, 20, 30, 35, 40, 45, and 50 DAF. Photographs showing the colors of the inner and outer sections of the fruits were taken during low-temperature storage (4°C) and after transfer to shelf conditions (20°C). The seeds of the fruit harvested 15 DAF showed 100% browning within 7 d of low-temperature storage (CI index, 4.0). In contrast, only 10% of the peppers harvested 35 DAF displayed brown seeds (CI index, 0.4). There was no significant difference in CI index between 35 DAF and 50 DAF seeds following low-temperature storage (4°C), and after transfer to shelf conditions (20°C). Similarly, seed browning in hot pepper was severe in fruit stored at 5°C, but only in fruit harvested 15 DAF [5]. In the present study, seed browning rates in the chilled samples exhibited strong linear correlations with storage period and harvest time (Figure 1). The CI index in seeds of fruits harvested from 15 DAF to 30 DAF was significantly higher than in fruits harvested from 35 DAF to 50 DAF when fruits were stored for 15 days at 4°C and 4 days at 20°C (Fig. 1C). In addition, pepper fruit harvested from 15 DAF to 30 DAF exhibited seed browning without ripening progress until 15 days of cold storage (Fig. 1A), which is consistent with the finding of a previous study in which pepper fruit stored at 2°C exhibited seed browning without ripening

progress for 25 d [10]. Therefore, the results suggest that harvesting time of hot pepper “Nockgwang” significantly influences seed browning, and harvesting pepper after 35 DAF can suppress seed browning under long-term low-temperature storage (4°C).

3.3 Antioxidant capacity, total phenol content

Cell membranes are particularly susceptible to CI [29], as cold stress induces the generation of ROS associated with seed browning in hot pepper [20]. Here, we measured antioxidant activity in hot pepper seeds and pericarps at various developmental stages using the ABTS and DPPH assays (Tables 2 and 3). Antioxidant activity was the highest in 15 DAF seeds, but steadily declined thereafter, until 50 DAF. The seeds in fruit harvested 15 DAF had high antioxidant content early in storage but relatively low antioxidant levels under shelf conditions following low-temperature storage (Table 2). Under shelf conditions (20°C), after 15 d of low- temperature storage, the antioxidant levels detected based on DPPH were significantly higher 35 DAF than at other maturity stages (Table 2). The antioxidant activity in the seeds of fruit harvested 15 DAF was significantly higher than in seeds of fruit harvested 40–50 DAF, under 7-d low-temperature storage, with the seeds in fruit harvested 15 DAF exhibiting 100% browning (CI index, 4.0). In contrast, the seeds in fruit harvested 40–50 DAF exhibited no browning (Figure 2). Therefore, the antioxidant activity in the seeds did not prevent browning entirely during low-temperature storage.

Antioxidant activity is positively correlated with vegetable freshness. Oxidative stress responses are associated with the emergence of CI symptoms such as pepper fruit pitting [2]. Here, we compared antioxidant activity in the pericarp and seeds. The pericarp of fruits harvested 35–50 DAF exhibited relatively higher antioxidant activity than those of the pericarp of fruits harvested 15 DAF. Fruit harvested 50 DAF had the highest ABTS and DPPH content (Table 2). The findings are consistent with those reported by Jang et al. [30], who demonstrated that antioxidant activity 43–48 days post-anthesis (DPA) was similar to those in earlier stages. The response could explain why the seeds did not brown during cold storage.

Table 2. Changes in 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and total phenolic content in hot pepper seeds during storage.

	0 d	7 d	15 d	15 + 4 d
	DPPH (Trolox equivalent, $\mu\text{mol/g DW}$)			
15 DAF	5.19 ± 0.07 Aa	4.79 ± 0.10 Db	4.76 ± 0.02 Cb	3.64 ± 0.05 Cc
20 DAF	5.13 ± 0.08 Ab	5.42 ± 0.03 Aa	5.20 ± 0.02 Ab	3.18 ± 0.04 Dc
25 DAF	4.59 ± 0.09 Cb	5.01 ± 0.02 Ca	4.94 ± 0.04 Ba	3.73 ± 0.02 Cc
30 DAF	4.60 ± 0.06 Cc	5.23 ± 0.02 Ba	4.94 ± 0.02 Bb	3.90 ± 0.03 Bd
35 DAF	4.93 ± 0.04 Bb	5.08 ± 0.03 Ca	4.85 ± 0.03 BCb	4.18 ± 0.07 Ac
40 DAF	3.88 ± 0.02 Eb	4.44 ± 0.07 Ea	4.44 ± 0.06 Da	3.98 ± 0.03 Bb
45 DAF	4.36 ± 0.04 Da	4.03 ± 0.03 Fc	4.20 ± 0.03 Eb	3.90 ± 0.06 Bc
50 DAF	3.89 ± 0.05 Eb	4.04 ± 0.04 Fa	3.93 ± 0.04 Fab	3.99 ± 0.04 Bab
	ABTS (Trolox equivalent, $\mu\text{mol/g DW}$)			
15 DAF	15.78 ± 1.09 ABa	15.65 ± 1.05 Ca	13.33 ± 0.72 CDa	14.02 ± 0.91 ABa
20 DAF	17.40 ± 1.15 Ab	21.16 ± 0.61 Aa	17.50 ± 0.73 Ab	12.13 ± 0.67 Cc
25 DAF	14.69 ± 0.95 Bb	17.60 ± 0.36 Ba	17.12 ± 0.50 ABa	14.71 ± 0.34 Ab
30 DAF	16.96 ± 0.58 ABab	18.39 ± 0.73 Ba	16.35 ± 0.57 ABb	14.08 ± 0.49 ABc
35 DAF	14.97 ± 0.78 ABa	15.58 ± 0.72 Ca	15.18 ± 0.74 BCa	12.77 ± 0.53 BCb
40 DAF	7.33 ± 0.43 Db	11.11 ± 0.46 Da	11.62 ± 1.00 Da	8.50 ± 0.60 Db
45 DAF	10.94 ± 0.58 Ca	9.53 ± 0.47 Dab	8.06 ± 0.57 Eb	7.89 ± 0.63 Db
50 DAF	8.55 ± 0.62 Dab	9.29 ± 0.47 Da	7.29 ± 0.62 Eb	8.69 ± 0.64 Dab
	Total phenolics (gallic acid equivalent, mg/g DW)			
15 DAF	4.66 ± 0.01 Aa	4.57 ± 0.00 Ab	4.53 ± 0.00 Ac	3.33 ± 0.00 Ad
20 DAF	3.67 ± 0.02 Bb	4.01 ± 0.01 Ba	4.04 ± 0.04 Ba	2.66 ± 0.01 Bc
25 DAF	2.65 ± 0.02 Db	2.56 ± 0.01 Cc	3.41 ± 0.01 Ca	2.54 ± 0.01 Cc
30 DAF	2.73 ± 0.02 Ca	2.44 ± 0.00 Db	2.46 ± 0.01 Db	2.20 ± 0.00 Dc
35 DAF	1.88 ± 0.02 Ec	2.22 ± 0.01 Eb	2.29 ± 0.01 Ea	1.86 ± 0.01 Ec
40 DAF	1.11 ± 0.01 Gd	1.52 ± 0.01 Fb	1.66 ± 0.01 Fa	1.31 ± 0.01 Fc
45 DAF	1.54 ± 0.01 Fa	1.20 ± 0.01 Gb	1.15 ± 0.00 Gc	1.21 ± 0.01 Gb
50 DAF	1.12 ± 0.01 Ga	1.10 ± 0.02 Hab	1.12 ± 0.01 Ga	1.08 ± 0.01 Hb

*Fruits were harvested 15, 20, 25, 30, 35, 40, 45, and 50 days after flowering (DAF) and stored for 15 d at 4°C (15 d) and then for 4 d at 20°C (15 + 4 d). Means with the same uppercase letter in a column or same lowercase letter in a row are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Tissue browning induced by CI is associated with polyphenol oxidase and phenylalanine ammonia-lyase production. Such enzymes participate in phenolic biosynthesis and polymerization [5,31]. The total phenolic levels in the seeds of fruit harvested 15 DAF were significantly higher than in those harvested at any other stage under shelf conditions. In other words, brown seeds in fruit harvested 15 DAF had comparatively higher total phenolic content than those of non-browning seeds in fruit harvested 50DAF during low-temperature storage (Table 3).

The above results suggest a significant correlation between the degree of seed browning at different harvest times and changes in total phenolic levels under low-temperature storage.

Table 3. Changes in 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and total phenolic content in pepper pericarp during storage.

	0 d	7 d	15 d	15 + 4 d
DPPH (Trolox equivalent, $\mu\text{mol/g DW}$)				
15 DAF	28.74 \pm 1.21 Da	26.05 \pm 0.68 Cb	20.08 \pm 1.00 Ec	29.17 \pm 0.52 Da
20 DAF	35.96 \pm 1.16 BCa	27.01 \pm 0.39 Cb	29.70 \pm 1.79 Cb	28.03 \pm 0.42 Db
25 DAF	34.43 \pm 0.29 Ca	28.23 \pm 0.87 BCb	28.23 \pm 0.95 Cb	36.06 \pm 0.54 Ba
30 DAF	24.89 \pm 0.33 Ec	27.58 \pm 0.46 BCb	24.68 \pm 0.35 Dc	32.58 \pm 0.95 Ca
35 DAF	23.67 \pm 0.56 Ec	26.97 \pm 0.43 Cb	22.24 \pm 0.31 DEc	31.65 \pm 0.77 Ca
40 DAF	27.72 \pm 0.98 Da	22.13 \pm 0.94 Db	27.93 \pm 0.65 Ca	29.40 \pm 0.34 Da
45 DAF	37.09 \pm 0.49 Bab	30.27 \pm 1.60 Bc	38.02 \pm 0.42 Ba	35.30 \pm 0.50 Bb
50 DAF	42.64 \pm 0.81 Ab	39.99 \pm 1.29 Ac	47.83 \pm 0.51 Aa	40.61 \pm 0.68 Abc
ABTS (Trolox equivalent, $\mu\text{mol/g DW}$)				
15 DAF	42.49 \pm 1.44 Bb	30.51 \pm 0.49 Gc	27.62 \pm 0.19 Fd	45.44 \pm 0.53 Ca
20 DAF	45.40 \pm 1.47 Ba	38.69 \pm 0.11 Eb	45.98 \pm 0.54 Ca	44.29 \pm 0.30 Ca
25 DAF	35.67 \pm 1.53 Cc	45.04 \pm 0.52 Cb	46.49 \pm 0.26 Cb	57.53 \pm 0.12 Aa
30 DAF	33.58 \pm 0.61 Cd	46.13 \pm 0.24 Bb	37.20 \pm 0.57 Ec	52.33 \pm 0.35 Ba
35 DAF	34.02 \pm 1.96 Cc	38.38 \pm 0.13 Eb	36.31 \pm 0.33 Ebc	44.07 \pm 0.56 Ca
40 DAF	34.98 \pm 1.76 Cb	34.24 \pm 0.18 Fb	41.89 \pm 0.50 Da	40.78 \pm 0.52 Da
45 DAF	42.87 \pm 0.83 Bb	41.69 \pm 0.39 Db	51.22 \pm 0.32 Ba	50.87 \pm 0.89 Ba
50 DAF	50.53 \pm 0.81 Ab	50.58 \pm 0.34 Ab	55.76 \pm 0.11 Aa	51.80 \pm 0.65 Bb
Total phenol (gallic acid equivalent, mg/g DW)				
15 DAF	3.71 \pm 0.13 Cab	3.46 \pm 0.01 Eb	3.70 \pm 0.12 Dab	3.82 \pm 0.03 Da
20 DAF	3.91 \pm 0.05 Ba	3.53 \pm 0.01 Dec	3.76 \pm 0.01 Db	3.42 \pm 0.02 Fd
25 DAF	3.71 \pm 0.01 Cc	3.79 \pm 0.02 Cb	3.82 \pm 0.01 Db	4.42 \pm 0.02 Aa
30 DAF	3.23 \pm 0.02 Dd	4.00 \pm 0.05 Ab	3.46 \pm 0.00 Ec	4.24 \pm 0.01 Ba
35 DAF	3.31 \pm 0.01 Dc	3.59 \pm 0.02 Db	3.13 \pm 0.02 Fd	3.88 \pm 0.02 Ca
40 DAF	3.37 \pm 0.01 Dc	3.39 \pm 0.02 Fc	4.18 \pm 0.02 Ca	3.65 \pm 0.01 Eb
45 DAF	3.67 \pm 0.06 Cd	3.93 \pm 0.01 Bc	4.39 \pm 0.01 Ba	4.27 \pm 0.02 Bb
50 DAF	4.19 \pm 0.01 Ac	4.06 \pm 0.02 Ad	4.75 \pm 0.02 Aa	4.41 \pm 0.00 Ab

*Fruits were harvested at 15, 20, 25, 30, 35, 40, 45, and 50 days after flowering (DAF) and stored for 15 d at 4 °C (15 d) and for 4 d at 20 °C (15 + 4 d). Means with the same uppercase letter in a column or same lowercase letter in a row were not significantly different at $P < 0.05$ according to Duncan's multiple range test.

3.4. Analysis of the expression levels of antioxidant- and cell wall-related genes during storage

To confirm the relationships observed between seed browning and antioxidant levels during cold storage, we investigated the levels of expression of antioxidant-related genes in seeds of hot pepper. Gene expression levels of catalase (CAT) and peroxidase (POD) were significantly upregulated in the seeds of fruit harvested 15 DAF and 25 DAF, when compared to the levels in those harvested 35 DAF and 45 DAF, under 7 days of cold storage (Figure 2). The findings corroborate those for antioxidant levels in the seeds evaluated using ABTS and DPPH assays (Table 2). However, according to Boonsiri et al. [5], CAT and POD activity in seeds of fruits harvested 25 DAF was significantly higher than in seeds from fruit harvested 15 DAF, suggesting a negative correlation between browning and CAT and POD activity. Lee et al. [20] showed that genes related to superoxide dismutase, CAT, and ascorbate peroxidase were significantly upregulated in hot pepper seeds at 20 days and 2°C, when seed browning was severe. The results of the present study revealed antioxidant gene expression in immature seeds, indicating that seed browning during cold storage would be more severe in immature seeds than in mature seeds.

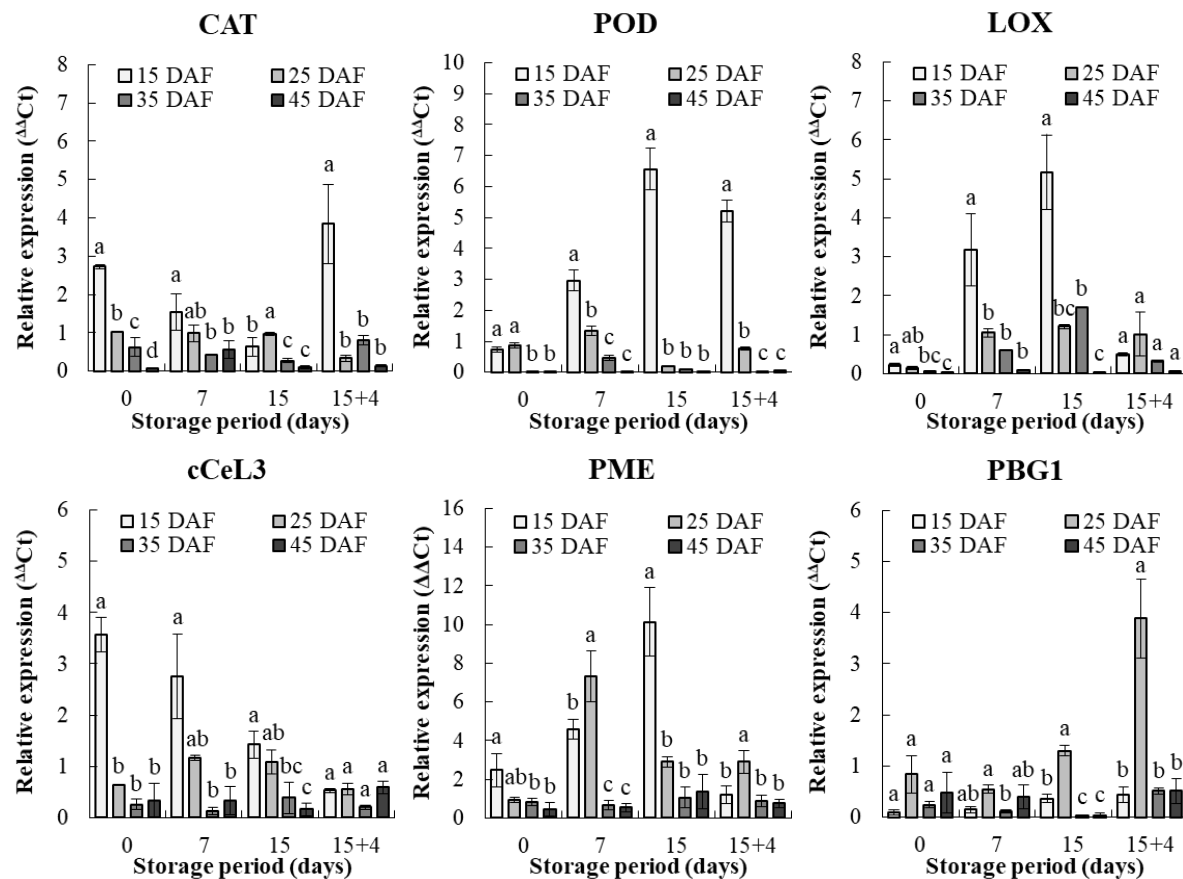


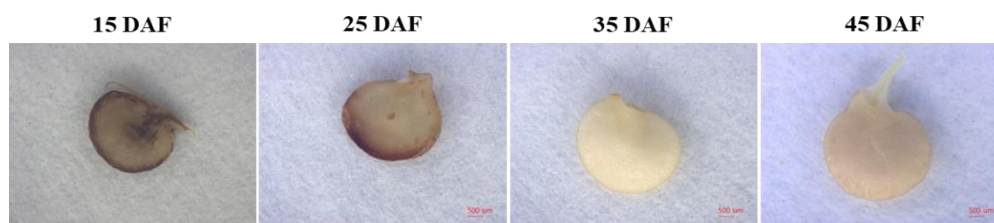
Figure 2. RT-qPCR analysis of the transcript levels of catalase (CAT), lipoxygenase (LOX), and peroxidase (POX) (upper panel), endo- β -1,4-glucanase (cCeL3), pectin methylesterase-like protein (PME), and β -Galactosidase-1 (PBG1) in seeds of hot pepper. Data are means \pm SE of three replicates. Different letters indicate significant differences among treatments within the same storage time according to the Duncan's New Multiple Range Test (DMRT) ($P < 0.05$). *Fruits were harvested 15, 25, 35, and 45 days after flowering (DAF) and stored for 15 d at 4°C and for 4 d at 20°C.

Seed browning might be associated with cell membrane peroxidation by LOX [20]. In the present study, LOX expression in the seeds of fruit harvested 15 DAF was significantly higher than in seeds of fruit harvested later (35 DAF to 45 DAF) during low-temperature storage. Here, we confirmed that cold stress induced LOX, which caused membrane lipid peroxidation, disrupted membrane integrity, and induced seed browning under low-temperature storage. We also examined the expression of cell wall-related genes during pepper fruit storage. The expression of pepper endo- β -1,4-glucanase (cCeL3) was significantly upregulated in seeds harvested 15 DAF than in seeds of fruits harvested at later stages, during low-temperature storage. However, enzyme activity had the lowest initial value in fruits harvested ~35 DAF. Pepper cCeL3 is involved in leaf abscission [32]. Endo-1,4- β -glucanase (EGase) is upregulated during fruit ripening and requires cooperative cell wall component disassembly during ripening and abscission [33]. The upregulation of cCeL3 in the seeds of fruit harvested 15 DAF might be associated with the softening of young seeds as EGase mediates fruit softening [34]. Seeds in fruit harvested 15 DAF were softer than those in fruit harvested 35 DAF and 45 DAF. PME1 expression in the seeds of fruit harvested 15 DAF and 25 DAF

was significantly higher than in seeds of fruit harvested 35 DAF and 45 DAF, during cold storage. PME hydrolyzes pectin, which is the primary constituent of the middle lamella and the primary cell wall. The expression of *PME1* in the seeds of fruit harvested 15 DAF and 25 DAF increased under low-temperature storage (Figure 2). Similarly, in sweet pepper, *PME1* activity progressively increased during storage [27]. Pepper β -galactosidase-1 (PBG1) gene expression in seeds of fruit harvested 25 DAF has been reported to be significantly higher than in 35 DAF and 45 DAF fruits, following 15 days of cold storage, and even after transfer to 20°C. PBG1 gene participates in fruit ripening in bell pepper [35]. Therefore, young seeds, which are sensitive to cold stress and chilling, may initiate cell wall degradation. Similarly, *PME1* upregulation may cause cell wall softening. Pectinesterase and β -galactosidase have been associated with CI sensitivity and loss of firmness in carambola fruit [36].

3.5 Anatomical analysis

In the present study, we explored the relationship between hot pepper seed maturity and CI by examining cell wall integrity after cryopreservation of seeds harvested at various times. Stereomicroscope images of seeds of pepper fruit harvested 15, 25, 35, and 45 DAF are shown in Figure 3A. After 7 d cold storage, the seeds of fruit harvested 15 DAF and 25 DAF browned. In contrast, the seeds of fruit harvested 35 DAF and 45 DAF exhibited no browning. We used SEM and LM to observe the structures of seeds obtained from fruits harvested at various times (Figure 3). The SEM images showed that the seed coat did not separate from the endosperm in the seeds of fruit harvested 25 DAF. These seeds exhibited browning after 7 d of storage at 4°C. In contrast, seeds from fruit harvested 35 DAF showed no browning; however, their seed coats were separated from their endosperms. The LM images revealed that the seeds from fruit harvested 35 DAF had fully developed seed coats and embryos. However, seeds from fruit harvested 25 DAF had damaged cells, only partially formed seed coats, and exhibited high sensitivity to cold storage. An earlier study reported that the early stages of pepper seed development were highly sensitive to high temperatures after anthesis. Hence, heat stress may have a detrimental effect on embryo formation [37].



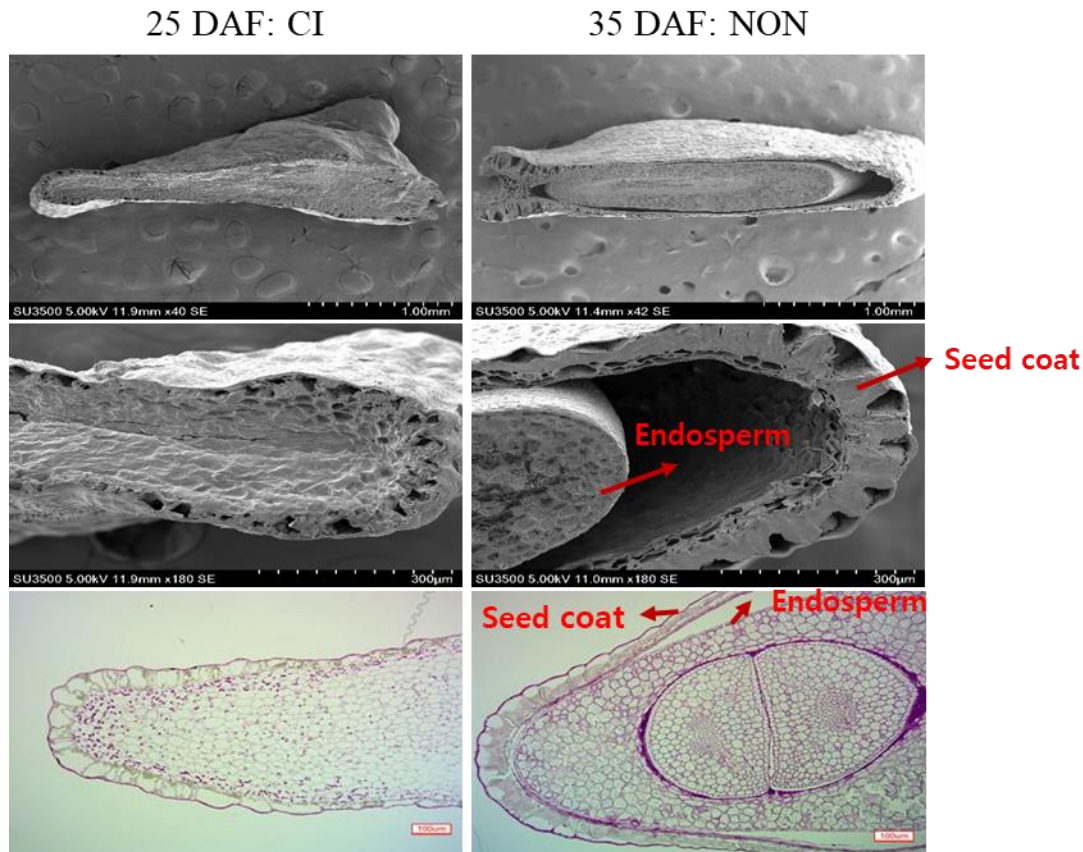


Figure 3. Pepper seed anatomy. A. Stereomicroscope image of pepper seeds. Fruits were harvested 15, 25, 35, and 45 days after flowering (DAF) and stored for 7 d at 4°C. B. Scanning electron microscope (SEM) images (upper pane) and light microscope (LM) images (lower panel) of pepper seed. Left: browning of seed in fruit harvested 25 DAF. Right: normal seed in fruit harvested 35 DAF. Fruits were harvested 25 DAF or 35 DAF and stored for 7 d at 4°C.

The results of the present study indicate that the degree of pepper seed browning varies with harvest time after flowering and is associated with changes in seed physicochemical properties. Seeds from fruit harvested after 35 DAF did not undergo browning. The seed coat may harden as the seeds mature after flowering. This change may confer physical resistance to cold stress. The above finding is consistent with the observation that chilled pepper fruit display visible symptoms of cell damage [15]. Similarly, in pea, the seed coat confers protection against CI [38].

4. Conclusion

The present study investigated the optimal harvest time that would minimize hot pepper seed browning induced by low-temperature storage. Harvesting pepper fruit 35 DAF resulted in excellent fruit quality and prevented seed browning. In contrast, the seeds of fruits harvested at 15 DAF had relatively higher antioxidant levels than those harvested later. Nevertheless, 100% of these seeds had turned brown within 7 d of cold storage. Cell wall degradation-related genes were upregulated and the seed coat was not fully developed until 25 DAF. Consequently, before seed maturity, the seeds were highly sensitive to chilling stress. However, the seeds of fruits harvested 35 DAF had fully formed seed coats. The primary

reason why the seeds of fruits harvested 35 DAF did not turn brown during cold storage was a mature hard seed coat conferred resistance to cold stress. This study confirmed that seed maturity in pepper was highly correlated with seed browning. Therefore, antioxidant activities in seeds are not responsible for the browning that occurs in the seeds of immature pepper fruit subjected to low-temperature storage. The results of the present study suggest that producers could minimize postharvest losses in hot pepper production that occur during long-term storage under low temperatures, and could facilitate the maintenance of the quality of hot pepper over longer periods, particularly in the course of long-distance export activities that require low-temperature distribution.

Supplementary Materials: Table S1: Primer sequences of genes used in the present study

Author Contributions: E.-Y.Y., J.-S.L., G.-R.D., and M.-H.P., experimental data and methodology; M.-H.P., Y.-P. H., original draft preparation, supervision, project administration, and funding acquisition; and M.-H.P. writing—review and editing, visualization, and data curation.

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