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Browning of 'Empire' and 'Fuji' Apples as Affected by Antioxidant Activities

Seok-Kyu Jung and Hyun-Sug Choi *

Department of Horticulture, Daegu Catholic University, Gyeongsan-si, Gyeongbuk 38430, Korea; gentleman71@msn.com

* Correspondence: hchoiuark@gmail.com

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Abstract: Internal ethylene concentration (IEC) and activities of peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) were analyzed to evaluate their effects on browning in late-harvested 'Empire' apples (*Malus* × *domestica* Borkh.), during air storage at 0.5 °C for five months, and for 'Fuji' apples treated with 1-methylcyclopropene (1-MCP), stored for seven months. IEC remained higher in the 'Empire' apples compared to values observed in the 'Fuji' apples for up to three months in storage, although 1-MCP treatment reduced the IEC in both fruit cultivars. Antioxidant enzymes, such as PPO, POX, and PAL, mostly increased in the flesh and core tissues in both 1-MCP-treated apple cultivars, but were slightly higher in the 'Empire' apples between one and three months of storage time. Browning developed in the 'Empire' apples after three months of storage, with high susceptibility to incidences of browning observed on the 1-MCP-treated fruit.

Keywords: apples; browning disorder; peroxidase; phenylalanine ammonia-lyase; polyphenol oxidase

1. Introduction

'Empire' and 'Fuji' apples (*Malus* × *domestica* Borkh.) are grown in the Northeastern USA and Japan, respectively, according to the US Apple Association [1]. They are popular cultivars grown commercially in the USA for their sweet flavor and crisp and juicy taste. However, apples are susceptible to incidences of browning associated with O_2 and CO_2 concentrations in controlled atmosphere (CA) storage [2–6] during the fruit ripening period. Few scientific references are available concerning physiological disorders of 'Empire' apples during CA storage [7–12], with no information available for a comparison of 'Empire' and 'Fuji' apples held in long-term cold air storage, the most commonly used storage method in low and middle income countries (LMICs).

Browning in the apples in this study was characterized by phenolic content or by complex interactions between phenolic content and polyphenol oxidase (PPO) [13]. The extent of apple browning varied by cultivar, phenolic content, or by level of antioxidant enzymes such as PPO, peroxidases (POX), and phenylalanine ammonia-lyase (PAL), and was directly or indirectly associated with the production of IEC and oxidative stress in pre- and post-harvest [13–15]. Some researchers indicated that enzymatic browning in apples is caused by the oxidation of phenolic compounds to *o*-quinones by PPO synergistically reacting with POX [4,10,16], resulting in complex brown pigments [13,17].

Harvesting of apple fruit is often purposely delayed in many commercial orchards to avoid a large supply of shipments and to foster a marketing spread, resulting in reductions in fruit metabolic rates and increased incidences of storage disorders [1]. The late-harvested fruit should be treated with 1-MCP, widely used as an ethylene action inhibitor [18] and distributed with cold-chain systems to increase the shelf life of the fruits. However, 1-MCP-treated, CA-stored 'Empire' apples have shown browning susceptibility, speculated to have been induced from an increased insensitivity to ethylene

production and corresponding high potential of oxidative stress [5,7,9–12], with little known about the effect of 1-MCP and cold storage time on the enzymatic browning of the fruit.

The aim of this research was to investigate the variation of antioxidant enzymes and the subsequent influence of this variation on browning in late-harvested 'Empire' and 'Fuji' apples during cold storage, and to examine how other factors relating to the incidence of browning (such as cultivar, IEC, and antioxidant enzymes) influence the effects of 1-MCP and storage time to minimize storage disorders.

2. Materials and Methods

2.1. Plant Material and 1-MCP Treatment

'Empire' and 'Fuji' apples were harvested in the late stages of maturity from Cornell University orchards located in both Ithaca and Lansing, USA. Apples were pre-cooled in air storage at 2 °C for 24 h. 'Empire' and 'Fuji' apples were treated as follows: (1) Untreated and (2) treated with 1-MCP at 1.0 $\mu L \cdot L^{-1}$ using the method of Watkins et al. [19]. 'Empire' and 'Fuji' apples were monitored in air storage under normal atmosphere at 0.5 ± 0.1 °C with 95% relative humidity in two rooms with similar fan speed and performance, for periods of 5 and 7 months, respectively. At each sampling point, five individual fruits from both fruit cultivars were sampled to measure internal ethylene concentration (IEC), flesh, and core color. Flesh and core tissues of five sub-samples of 100 fruits each in both cultivars were sliced, immediately put into liquid nitrogen, and stored at −80 °C for analyses of peroxidase (POX), polyphenol oxidase (PPO), and phenylalanine ammonia-lyase (PAL) activity.

2.2. Internal Ethylene Concentration (IEC)

IEC was measured by a Hewlett Packard 5890 serious II gas chromatograph (Hewlett-Packard, Wilmington, MD, USA) equipped with a flame ionization detector and fitted with a stainless steel column packed with 60/80 mesh alumina F-1 (2 m × 2 mm, i.d.). Oven, injector, and detector temperatures of the gas chromatograph were 200, 220, and 250 °C, respectively. A total of 1.0 mL of gas sample from each individual apple was injected into a Hewlett Packard 5890 serious II gas chromatograph.

2.3. Extraction of Enzymes

'Empire' and 'Fuji' apples were randomly picked from storage at each sampling point. Three grams of samples from core and flesh tissues separately were ground to a fine powder in liquid nitrogen. The fine powder samples were put into 9.0 mL of extraction buffer consisting of a 200 mM phosphate buffer (pH 7.8) containing 2.0 mM ethylenediaminetetraacetic acid (EDTA), 5.0% polyvinylpolypyrrolidone (PVPP), and 1.0 mM phenylmethanesulfonylfluoride (PMSF). The mixture of buffer and sample were homogenized and centrifuged at 14,000× *g* for 30 min at 4 °C. The supernatant was stored for further use in assaying antioxidant enzymes and protein.

2.4. POX and PPO Activities

POX and PPO activities in flesh from both apple cultivars were assayed as described by the methods of Kochhar et al. [20]. For the measurements of the activities of POX and PPO, 200 µL of extraction was added to the different 2.8 mL assay solutions separately. The reaction mixture consisted of a 100 mM phosphate buffer (pH 6.8) with 2.7 mM guaiacol and 4.0 mM H_2O_2. The assay solution of PPO was a 100 mM citrate-200 mM phosphate buffer (pH 5.0) containing 50 mM catechol. The activity of POX and PPO was expressed in terms of enzyme units/mg tissue, with optical density (OD) at 470 and 420 $nm \cdot mg^{-1}$ of protein respectively, measured using a spectrophotometer (Beckman-Counter, DU 7400, Fullerton, CA, USA), with the protein concentration from the enzyme extract determined using Bradford's method [21].

2.5. PAL Activity

PAL activity was analyzed by conversion of L-phenylalanine into trans-cinnamic acid at 270 nm in a UV–Vis spectrophotometer. Homogenized samples were contained in 6 mL of extraction buffer [50 mM Tris-HCL buffer, pH 8.8, ß 2-mercaptoethanol, 5.0 mM EDTA, 5.0 mM ascorbic acid, 10 µM leupeptin, 1.0 mM PMSF, and 0.15% (*w*/*v*) PVP], and centrifuged at 12,000× *g* for 20 min at 4 °C. The volume of reaction mixture [16 mL L-phenylalanine, 50 mM Tris-HCL buffer (pH 8.8), 3.6 mM NaCl, and 0.5 mL of enzyme solution], which was made up to 3 Ml with deionized H_2O, kept for incubation at 37 °C for 2 h, mixed with 500 µL of 6.0 M HCl, recorded an increase in absorbance at 270 nm before and after incubation. The amount of PAL activity was expressed as µmol cinnamic acid h^{-1} mg^{-1} protein. Protein was estimated using BSA as a standard according to Bradford [21].

2.6. Measurements of Color and Browning Assessment

Flesh and core color in tissues of both cultivars was measured using a Minolta Chroma Meter (Model CR300, Minolta Co., Osaka, Japan) with an 8 mm measuring area. The 3-point flesh and core tissues from individual apples were measured by CIELAB, which expressed colors at L* (lightness), a* (red), and b* (yellow) values.

The incidence of browning at cut surfaces of flesh and core tissue was visually assessed by the extent of browning as follows [0 = 0% (no browning), 1 = 1–10% (slight browning), 2 = 11–25%, 3 = 26–75%, and 4 = 76–100% (almost complete browning)].

2.7. Statistical Analysis

All statistical data analyses were done with Minitab software v. 15.1 (Minitab, Inc., State College, PA, USA). Individual models were conducted for separate interaction factors (No treatment, 1-MCP, and period). The data were performed by one-way analysis of variance (ANOVA). Means were separated using least significant differences (LSD) at $p < 0.05$. Data over time are shown as means ± standard errors.

3. Results and Discussion

3.1. Internal Ethylene Concentration

'Empire' apples sharply decreased in IEC at one month after air storage, and remained between 15–16 µL·L^{-1} thereafter for untreated fruit and at less than 10 µL·L^{-1} for 1-MCP-treated fruit (Figure 1A). IEC values in untreated 'Empire' apples increased two months after air storage and three months after CA storage in previous reports [7,10,22]. Incompatibility in these results would reflect differences in fruit maturity [8], and the late-harvest time of the 'Empire' fruit obtained in this work might have caused gradual increases in internal fruit respiration, CO_2 injury, and enzymatic browning. On the other hand, IECs of untreated 'Fuji' apples gradually increased to 27.6 µL·L^{-1} up until 5 months of storage time due to the ripening process (Figure 1B) as previously reported in CA stored 'Fuji' apples [23]. 1-MCP is extensively used by agricultural industries to maintain storage potential and quality of apples during long-term storage through the blocking of the ethylene signal transduction pathway and inhibition of ethylene production [14,18]. 1-MCP-treated fruit showed lower levels of IEC than untreated 'Empire' and 'Fuji' apples throughout the air storage period ($p < 0.05$), which was in accordance with other findings in the various apple cultivars [24].

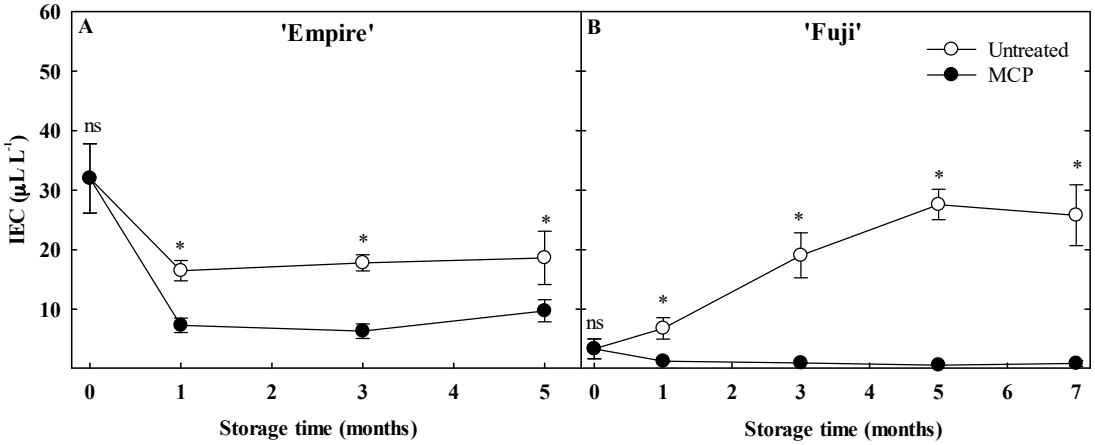

Figure 1. Internal ethylene concentration (IEC) in browning of 'Empire' (**A**) and 'Fuji' (**B**), either untreated or treated with 1 µL·L^{-1} 1-MCP, and stored at 0.5 °C in air storage for up to five and seven months, respectively. Bars represent error of the means (S.E.M; n = 5) when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively.

3.2. Antioxidant Enzymes

POX activity in the apples flesh tissue rapidly increased from 1.0 to 3.5 U mg·protein^{-1} in both untreated and 1-MCP-treated 'Empire' apples during air storage (Figure 2A), but remained at low levels, between 0.5–1.5 U mg·protein^{-1}, for 'Fuji' apples (Figure 2B). 1-MCP treatment significantly increased in 'Fuji' apples at one month of storage time, which is a similar result to increasing resistance to oxidative stress, whereas other antioxidants, superoxide dismutase or catalase, were not significantly different between the untreated and treated fruit [11,16,25,26]. The higher activity of POX in the 'Empire' apples compared to those of values observed on the 'Fuji' apples ($p < 0.05$) was presumably due to high POX occurring in both untreated and treated fruit. The increased POX activities reflected a higher potential toward diffuse flesh discoloration, alleviating the development of storage disorder and flesh browning [15]. POX activity in the core tissue fluctuated during storage for 'Empire' apples (Figure 2C) and 'Fuji' apples (Figure 2D). 1-MCP slightly increased the POX activity in the core tissue of 'Empire' and of 'Fuji' apples during storage, as observed in the flesh tissue.

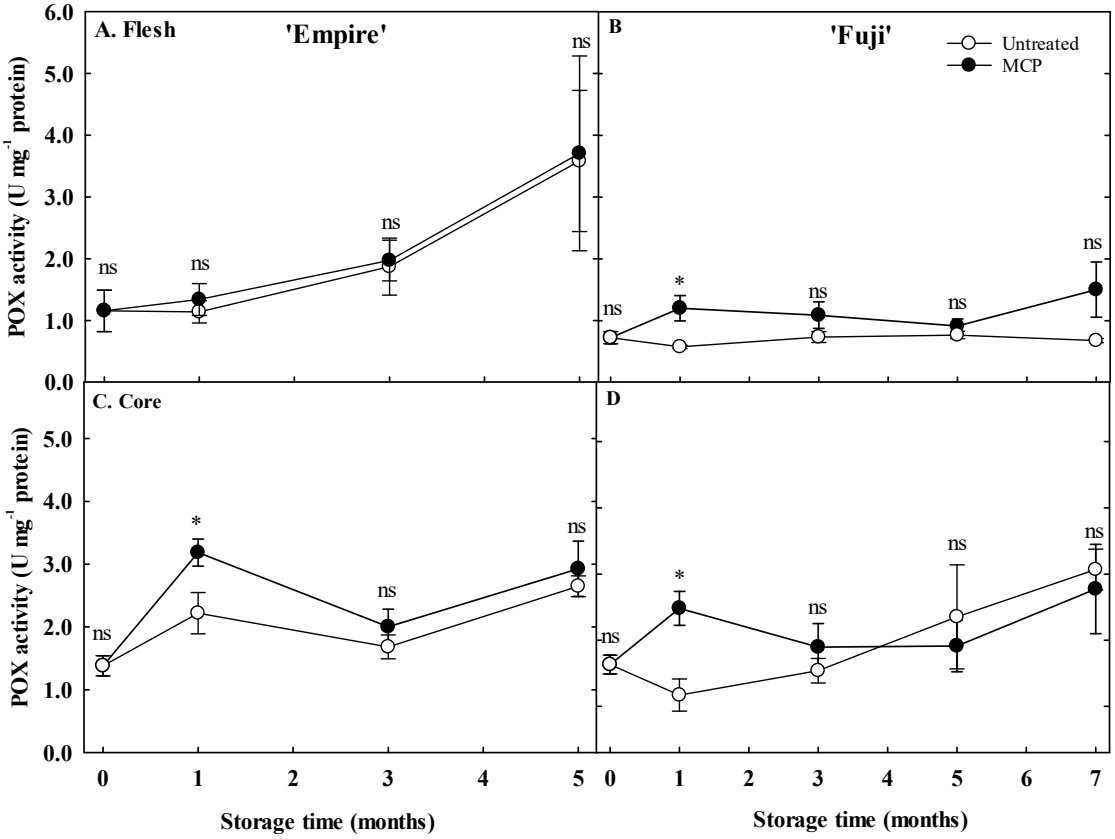

Figure 2. POX (Peroxidase) in flesh (**A**,**B**) and core tissue (**C**,**D**) on 'Empire' and 'Fuji', either untreated or treated with 1 μL·L^{-1} 1-MCP, and stored at 0.5 °C in air storage for up to five and seven months, respectively. Bars represent error of the means (S.E.M; $n = 5$) when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively.

PPO activities in the flesh and core tissues fluctuated for both apple cultivars during storage, and was slightly higher in the 'Empire' apples up to three months after storage compared to those of 'Fuji' (Figure 3A–D). PPO activity in 'Empire' apples stored in CA for a 10.5-month period was stimulated by 1-MCP treatment [10], which was not clearly observed in PPO of flesh and core tissues in 'Empire' apples treated with 1-MCP. PPO activities in core tissue of 'Empire' apples decreased from one month to three months storage, presumably contributing to the same browning of the fruit as observed in the CA-stored 'Empire' apples [10].

PAL is a key enzyme of phenolic metabolism producing chlorogenic acid and caffeic acid derivatives; however, the relationship between enzyme activity and browning is mostly highlighted not in apples [14] but in cut lettuce and other fresh-cut fruit and vegetables. 1-MCP treatment increased PAL activity in the flesh tissue of 'Empire' apples during three months of air storage but decreased after five months of storage, compared to values observed for untreated fruit (Figure 4), presumably due to responses to the production of IEC (Figure 1A).

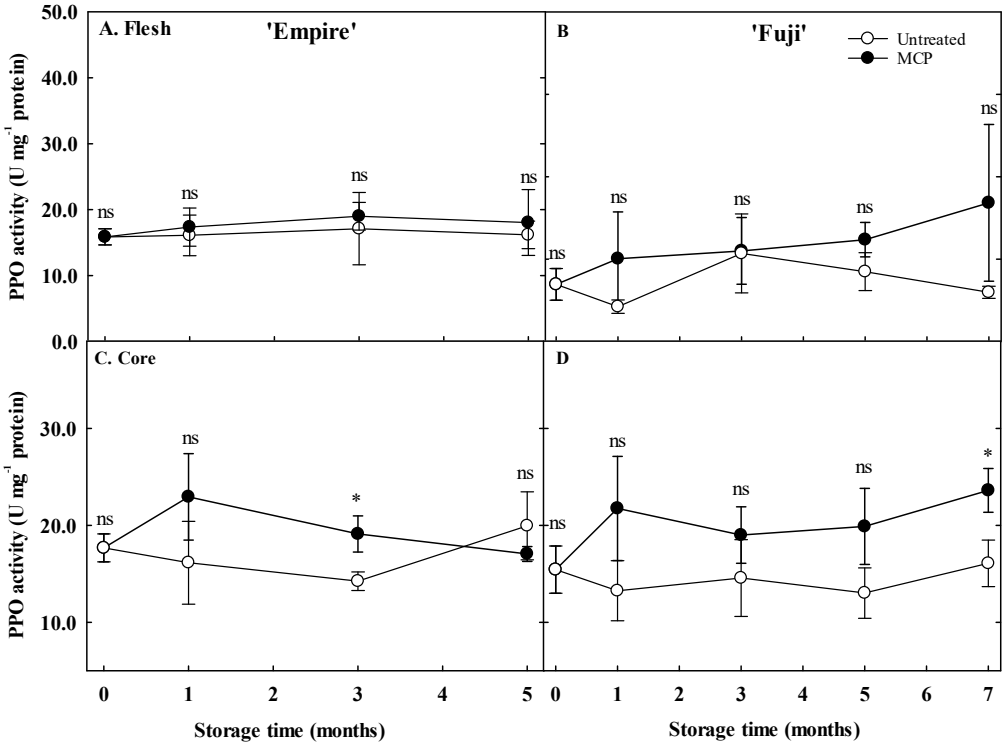

Figure 3. PPO (Polyphenol oxidase) in flesh (**A**,**B**) and core tissue (**C**,**D**) on 'Empire' and 'Fuji' apples, either untreated or treated with 1 μL·L^{-1} 1-MCP, and stored at 0.5 °C in air storage for up to five and seven months, respectively. Bars represent error of the means (S.E.M; n = 5) when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively.

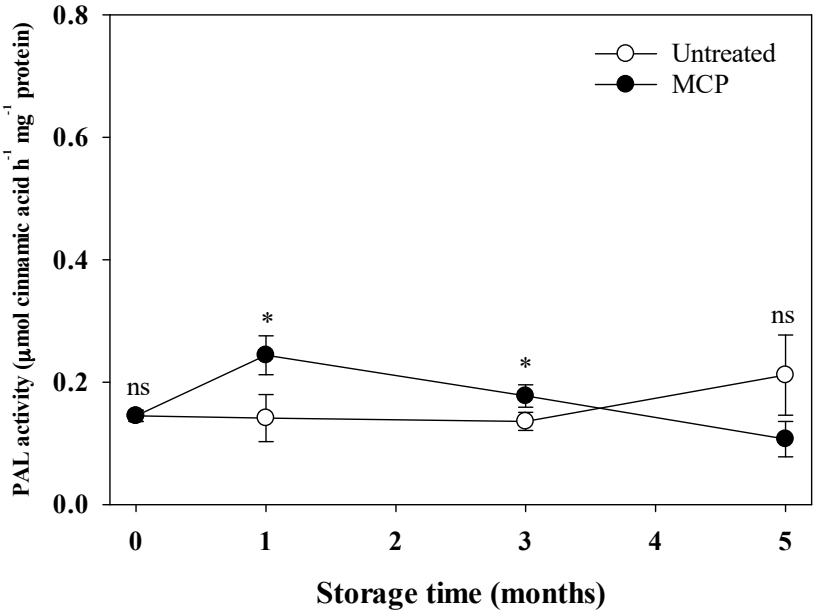

Figure 4. PAL (Phenylalanine ammonia lyase) activity in flesh tissue on 'Empire' apples, either untreated or treated with 1 μL·L^{-1} 1-MCP, and stored at 0.5 °C in air storage for up to 5 months. Bars represent error of the means (S.E.M; n = 5), when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively.

3.3. Browning and Color Value

Incidences of flesh browning started to be detected in both untreated and 1-MCP-treated 'Empire' apples at three months in air storage (Figure 5A). IEC might indirectly and directly influence secondary metabolism and flesh browning [27], which did not appear until six months of air storage in 'Empire' apples at early and late harvests with low IEC [5]. In our experiment, high IEC levels of 32.0 $\mu L \cdot L^{-1}$ in the initial periods of storage for 'Empire' apples would have contributed more to the incidence of browning, with no symptom occurring for 'Fuji' apples with low IEC levels of 3.3 $\mu L \cdot L^{-1}$ (Figure 5B). Additionally, 'Empire' apples showed lower fruit density and higher gas diffusion rates across cortical tissue than those of 'Fuji' apples, which would have increased the susceptibility of 'Empire' to browning [3,5]. Briefly, internal browning of 'Empire' apples might involve high ethylene evolution and free radical damage to proteins at a late harvest, stimulating the activities of POX ($r^2 = 0.6263$) and PPO ($r^2 = 0.9992$) in the initial period of storage. The trend towards browning increased in the 1-MCP-treated 'Empire' apples, which might be ascribed to energy level changes caused by blocking the ethylene-signaling pathway and leading to increased insensitivity to ethylene production in the flesh tissue [10–12].

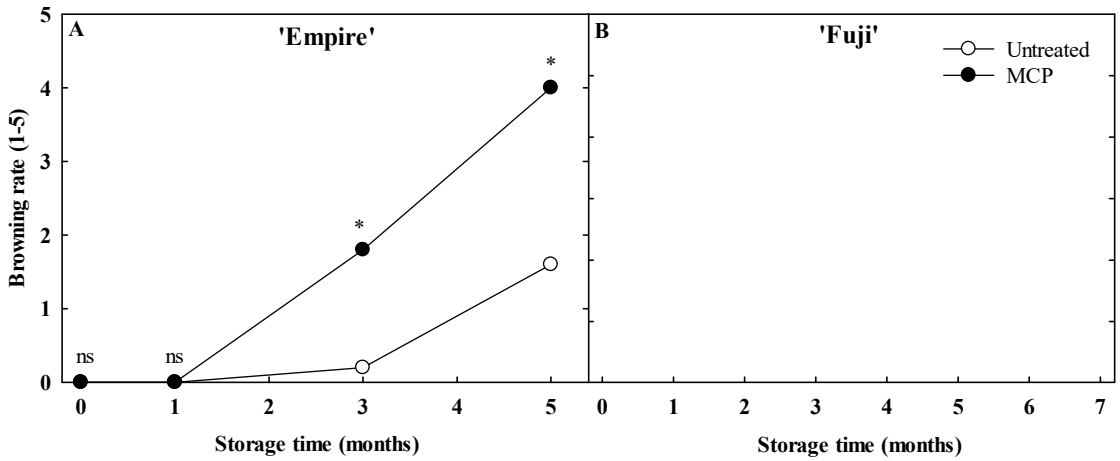

Figure 5. Incidence of browning of 'Empire' (**A**) and 'Fuji' (**B**) apples, either untreated or treated with 1 $\mu L \cdot L^{-1}$ 1-MCP, and stored at 0.5 °C in air storage for up to five and seven months, respectively. Bars represent error of the means (S.E.M; $n = 5$) when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively

In 'Empire' apples, L* values in flesh tissue in both untreated and 1-MCP-treated fruit increased until one month after air storage and then started to decline by the end of the storage period ($p < 0.05$; Figure 6A), with rising trend observed for a* (Figure 6C) and b* (Figure 6E) values. Increased browning of the 'Empire' apples would have resulted in a declined L* value, representing lightness on the fruit. In both untreated and 1-MCP-treated 'Fuji' apples, the L* value in flesh tissue slightly increased at one month of storage (Figure 6B), with little variation observed for a* (Figure 6D) and b* (Figure 6F) values. No treatment effects on the changes of value in L*, a*, and b* in the flesh tissue were detected until three month of the storage ($p > 0.05$). The value changes of L*, a*, and b* of core tissue in 'Empire' and 'Fuji' apples showed a similar response pattern to the flesh tissue (Figure 7A–F).

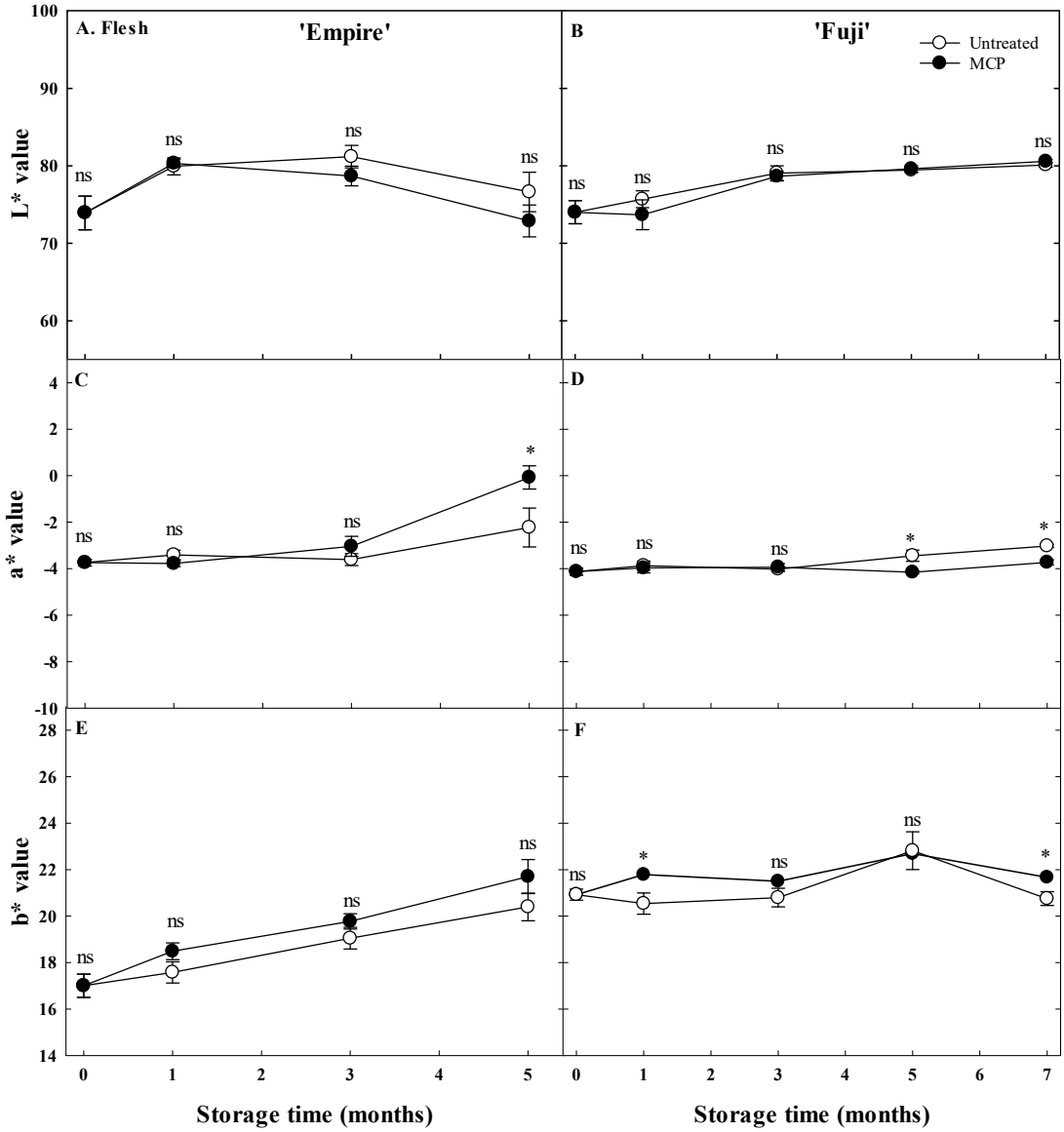

Figure 6. Color L* (lightness, **A**,**B**), a* (red, **C**,**D**), and b* (yellow, **E**,**F**) values (of flesh tissue on 'Empire' and 'Fuji' apples, either untreated or treated with 1 μL·L^{-1} 1-MCP, and stored at 0.5 °C in air storage for up to five and seven months, respectively. Bars represent error of the means (S.E.M; $n = 5$) when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively.

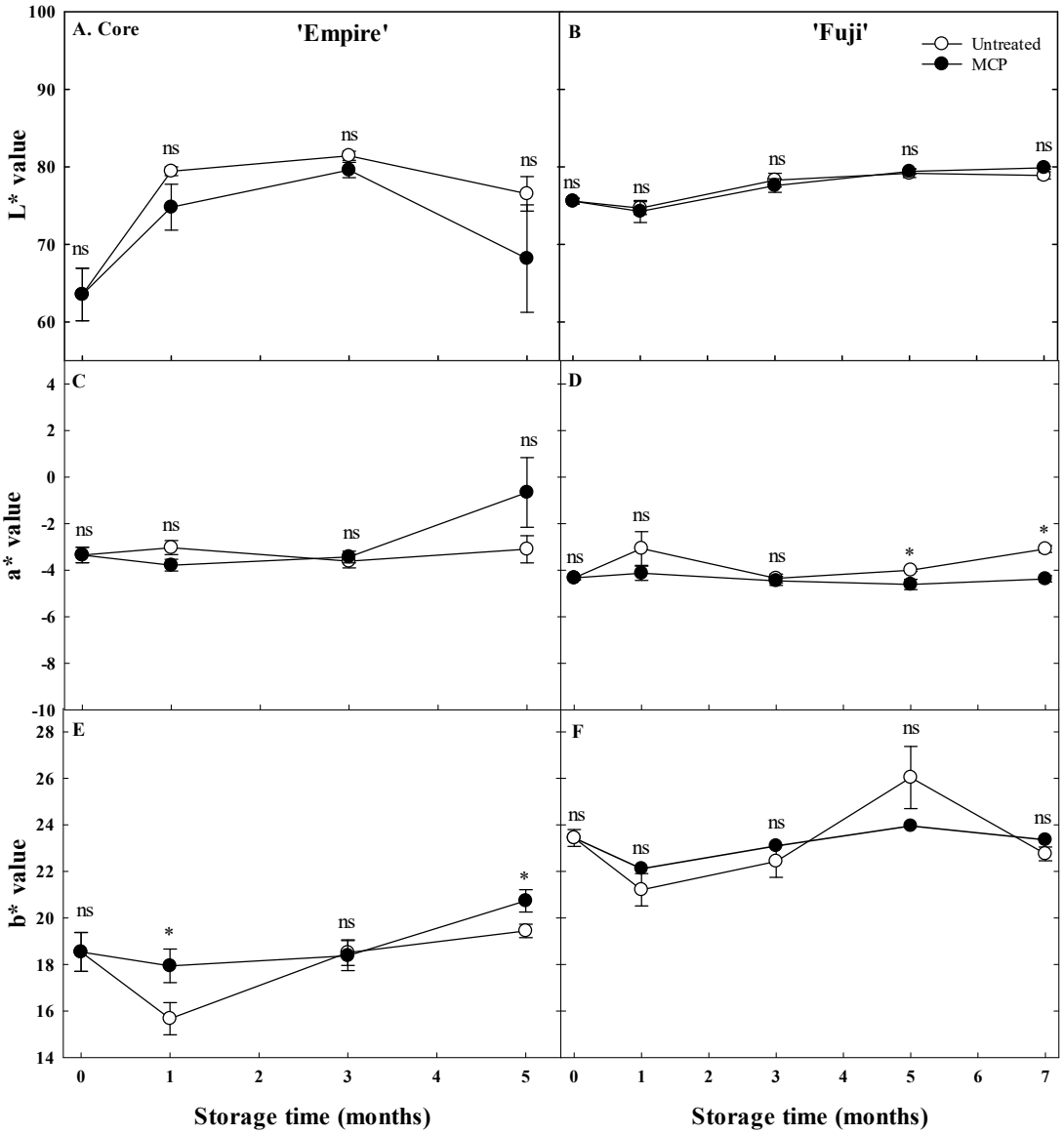

Figure 7. Color L* (**A**,**B**), a* (**C**,**D**), and b* values (**E**,**F**) of core tissue on 'Empire' and 'Fuji' apples, either untreated or treated with 1 μL·L^{-1} 1-MCP, and stored at 0.5 °C in air storage for up to five and seven months, respectively. Bars represent error of the means (S.E.M; n = 5) when larger than the dimension of the symbol. ns and * indicate non-significant and significant differences between untreated and 1-MCP treatments at $p < 0.05$, respectively.

4. Conclusions

The combination of late-harvested apples treated with 1-MCP and kept in long-term cold storage has been largely under-evaluated regarding its effectiveness in reducing enzymatic flesh browning, minimizing significant post-harvest losses, and maximizing growers' economic returns [28]. Understanding the relationship between IEC and antioxidant activity in flesh or core tissue could be an important factor in identifying cold-stored 'Empire' and 'Fuji' apples' susceptibility to browning. 1-MCP treatment potentially leads to oxidative stress and incidences of browning in 'Empire' apples during storage periods, which was not observed in the 'Fuji' apples, possibly due to their low IEC and CO_2 sensitivity associated with their structural rigidity, high fruit density, and late fruit cultivar. However, more work should be conducted with a consideration on pre-harvest factors, such as mineral nutrition, climate condition, and the fruit maturation stage [5,8], which all contribute to initial IEC, CO_2 sensitivity, and antioxidant activities.

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