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DYNAMICS OF ANTI-OXIDANT LEVELS AND ACTIVITIES OF REACTIVE OXYGEN-SCAVENGING ENZYMES IN 'PINK LADY' APPLE FRUIT DURING MATURATION AND RIPENING

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Abstract

'Pink Lady' apple fruit were evaluated for anti-oxidative stress at various stages of fruit maturity and ripening. Fruit were harvested at weekly intervals starting 7 days before anticipated commercial harvest up to 42 days after commercial harvest (DACH), and then fruit harvested at commercial maturity were allowed to ripen at 22 ± 0.5°C for 20 days. Ethylene production and respiration rate increased with advancing fruit maturity. Fruit firmness and titratable acidity (TA) decreased, whilst SSC and SSC:TA ratio increased with increase in fruit maturity and ripening. A significant decline in SOD (SOD; EC 1.15.1.1) activity in fruit harvested on 21 and 28 DACH was detected in fruit skin and pulp tissues and then SOD activity increased. During fruit ripening period, skin and pulp tissue also exhibited a similar trend in SOD activity. A significant increase in the activity of catalase (CAT, EC 1.11.1.6) enzyme was observed in the fruit skin and pulp tissue during fruit maturation and ripening. 'Pink Lady' apple fruit exhibited appreciable levels of SOD and CAT activities harvested during extended period of fruit harvest maturity and subsequent fruit ripening.

Introduction

Antioxidants are compounds capable of quenching biologically toxic reactive oxygen species (ROS) without undergoing conversion to harmful free radicals (Lurie, 2003). The most damaging free radicals are superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) (Elstner, 1987; Fridovich, 1988). ROS are generated in aerobic environments during normal cell metabolism, but high levels of these molecules can occur during oxidative stresses. Exposure of plants to extreme temperatures, water logging, ozone, sulphur dioxide, and pathogen infection have been reported to induce ROS formation (Bowler *et al*., 1992). ROS play an important role in senescence and ageing processes of plants (Masia, 1998; Yang *et al*., 2008). In humans ROS have been implicated in many age-related, neurodegenerative, cardiovascular and digestive diseases (Fridovich, 1995). Epidemiological studies have demonstrated that consumption of diets rich in fresh fruits and vegetables are associated with increased level of antioxidants with decreased risk of cardio- and cerebrovascular diseases and cancer mortality (Gil *et al*., 2000).

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Living organisms have evolved protective mechanisms to minimize the deleterious effects of free radicals. These anti-oxidant systems employ both enzymatic substances such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) enzymes and non-enzymatic substances such as glutathione, tocopherols, phenolics, flavonols, catechins, ascorbic acid and carotenoids. SOD detoxifies $O_2^{\cdot-}$ free radicals by converting them to O_2 and H_2O_2 (Monk *et al*., 1989; Tausz *et al*., 2004). SOD is ubiquitous in aerobic organisms in all subcellular compartments where oxidative stresses are likely to occur (Bannister *et al*., 1987; Fridovich, 1986; Halliwell & Gutteridge, 1989). H_2O_2 can lead to production of hydroxyl ($OH^{\cdot}$) free radical in Haber-Weiss reaction (Bowler *et al*., 1992). Removal of H_2O_2 is therefore, important in order to prevent its direct or indirect toxic effects. CAT is localized mostly in peroxisomes, catalyses H_2O_2 breakdown into H_2O and O_2 (Bowler *et al*., 1992; Singh & Kesavan, 1992). CAT also removes electrons (e^-), which can lead to the production of $O_2^{\cdot-}$ free radicals (Singh & Kesavan, 1992).

If free radicals are not inactivated, they induce lipid peroxidation (Dhindsa *et al*., 1981), which commences the deteriorative changes associated with fruit ripening (Du & Bramlage, 1994) and senescence (Du & Bramlage, 1995). The incidence of browning disorder in 'Braeburn' apple fruit has been found to be related with lower activities of SOD and CAT enzymes (Gong *et al*., 2001). Postharvest application of diphenyl amine has been reported to reduce the scald development with enhanced activities of SOD and CAT during low temperature storage in 'Red Spur Delicious' apple (Abbasi & Kushad, 2006). In apple early stages of maturity are important to the subsequent storage behaviour of the fruit and its postharvest life. In a previous work by Prior *et al*., (1998) it has been reported that blueberries harvested at advanced maturity stage yielded higher anti-oxidant, anthocyanin and total phenolics. Lentheric *et al*., (1999) claimed that delayed harvest decreased the activity of SOD and CAT in pear fruit.

Poor and erratic colour development in 'Pink Lady' apple causes serious economic losses to growers in Australia and rest of the world (Whale & Singh, 2007). 'Pink Lady' is an important Australian apple cultivar bred in Western Australia and commercially grown in North and South America, South Africa, Europe and New Zealand. Apple growers in Western Australia practice delayed harvesting to improve fruit colour in 'Pink Lady' apple, but this practice usually adversely affect storage life and fruit quality in controlled atmosphere and low temperature stored fruit. A burst in the ethylene production has been reported with onset of fruit ripening in 'Pink Lady' apple, consistent with a climacteric fruit Being climacteric (Whale & Singh, 2007). During fruit ripening, a rise in H_2O_2 and other ROS have been reported to be accompanied by parallel rise in ethylene production and activities of SOD and CAT enzymes in apple and pear (Brennan *et al*., 1977; Gong *et al*., 2001; Leshem *et al*., 1986b; Masia, 1998). Similarly in on-tree 'Fuji' and 'Golden Delicious' apples, the activities of CAT and SOD enzymes were correlated with climacteric ethylene peaks (Masia, 1998). Earlier Abassi *et al*., (1998) have reported the activities of active oxygen-scavenging enzymes such as SOD, peroxidase and CAT during flower bud development and flower maturation and as well as in immature 'Red Spur Delicious' apple fruit. However, no information is available on the activities of oxygen-scavenging enzymes during apple fruit maturation and ripening in 'Pink Lady' apple. The aim of this research work was to investigate changes in the ethylene production, respiration rate, activities of active oxygen-scavenging enzymes such as SOD and CAT as well as level of total antioxidants in skin and pulp tissues of the 'Pink Lady' apple fruit.

Materials and Methods

Plant material: Apple (*Malus domestica* Borkh cv. 'Pink Lady') fruit were harvested from 5 year old uniform trees grafted on MM 26 rootstock grown at the Karragullen, Perth Hills (lat. 31°57′S: log. 115°50′E), Western Australia. Trees were planted in north-south direction maintaining row distance of 3.7 m and plant distance of 1.5 m**,** trained on a palmette training system. Cultural practices, such as, nutrition, irrigation, pruning, thinning and plant protection control measures were carried out according to standard cultural practices (Mackey *et al.*, 1994).

Experiments: In first experiment, fruit were harvested starting 7 d before anticipated commercial harvest on 183 d after full bloom and continued at 7 d intervals up to 42 d after commercial harvest (DACH). Fruit maturity at commercial harvest was assessed on starch pattern index (SPI). Fruit were harvested when core showed clear ring and SPI was 3 using the guide lines detailed by Department of Agriculture Western Australian. Following harvest the respiration rate and ethylene production was estimated from the whole fruit, whilst activities of SOD, CAT enzymes, protein content as well as level of total antioxidants were determined in the fruit skin and pulp tissues. The experiment was in randomized complete block design using two trees as an experimental unit and three replications.

In the second experiment, the fruits were harvested at commercial maturity (190 d after full bloom) as explained in the first experiment. Following harvest fruit were ripened at 22 ± 0.5°C and 85% RH. The activities of SOD and CAT enzymes, protein content and level of total antioxidants in fruit skin and pulp tissues were determined at 5 d intervals up to 20 d. The experiment was laid out in a completely randomized design with 10 fruit as an experimental unit replicated three times.

Respiration rate: Respiration rate was determined as CO_2 produced from apple fruit. One fruit was sealed in a 1 L airtight jar fitted with a rubber septum for 1 h at room temperature (22 ± 0.5°C). Two-ml headspace gas sample was taken through rubber septum using a syringe and was injected into an infrared gas analyzer (Analyzer Series 1450 Food Package Analyzer; Servomex Ltd, East Sussex, England). Respiration rate of each sample was calculated based on the peak area of a 2-mL CO_2 standard (8.2% in N_2) obtained from BOC Gases (Perth, Australia) and was expressed as mmol CO_2 kg^{-1} h^{-1}.

Ethylene production: Ethylene production from the apple fruit was estimated using a gas chromatograph (6890 N Network GC system; Agilent Technologies, Palo Alto, CA, USA) fitted with a 2 m long stainless steel Supelco column (Porapak-Q 1/8", mesh size 80/100) and a flame ionization detector. The detailed procedure of ethylene estimation from apple has been reported earlier by Whale & Singh (2007) and ethylene production was expressed as nmol kg^{-1} h^{-1}.

Fruit firmness: Fruit firmness was determined by using an electronic pressure tester (model EPT-1 pressure tester, Lake City Technical Products Inc., Kelowna, BC, Canada) fitted with an 11 mm tip. A small slice of fruit skin was removed and firmness was recorded from two sides of individual apple fruit and means were expressed as newtons (N).

Soluble solids contents (SSC), titratable acidity (TA) and TSS:TA ratio: SSC of juice was determined using a digital refractometer (Atago-Palette PR 101, Atago Co., Itabashi-Ku, Tokyo, Japan) and was expressed as percent SSC as reported by Akhtar *et al*., (2010). To determine the TA, apple juice was titrated against 0.1 N NaOH to pH 8.2 using phenolphthalein as an indicator. TA was expressed as percent malic acid. The SSC: TA ratio was calculated by dividing SSC with corresponding TA value.

Preparation of cell free extracts for assay of enzyme: Fruit skin or pulp tissue (5 g) was taken from a pooled sample of 10 fruit, harvested from two trees in each replication. Fruit skin or pulp tissue was ground in 15 mL of 100 mM KPO_4 buffer (pH 7.8), containing 0.5% (v/v) Triton X-100 and 1 g (w/v) polyvinylpolypyrrolidone. The mixture was centrifuged at 15,000 x *g* at 4°C for 45 min. Following centrifugation the supernatant was collected and stored at –80°C for estimation of activities of enzymes and protein content.

Supseroxide dismutase (SOD) assay: The activity of SOD in fruit skin or pulp tissue was assayed spectrophotometrically by measuring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT), as reported by Abassi *et al*., (1998) with slight modification in the amount of crude extract used from pulp, which were 0, 400, 600, 800 and 1000 µL in reaction and control cuvettes to determine the SOD activity. The absorbance of the irradiated reaction mixtures at 560 nm were compared to the non-irradiated mixtures using a UV-vis Spectrophotometer (Model 6405, Jenway Ltd. Felsted, Dunmow, Essex, England) and per cent inhibition of colour was plotted as a function of the volume of enzyme extract according to Wang & Zheng (2005). From the resultant graph the volume of enzyme extract corresponding to 50% inhibition of the reaction was calculated and considered as one enzyme unit (Beauchamp & Fridovich, 1971). SOD activity was expressed as enzyme unit activity mg^{-1} protein of pulp or skin.

Catalase (CAT) assay: CAT activity in fruit skin and pulp was assayed by using method of Luck (1965). The reaction was carried out using two buffer solutions. Buffer-A consisted of 15 M KPO_4 buffer (pH 7.0) while the Buffer-B consisted of 1.25 10^{-2} M H_2O_2 in 15 M KPO_4 buffer (pH 7.0). The enzyme extract (50 to 100 µL) was added to each of two cuvettes, one containing 1 mL of buffer-A and the other containing 1 ml of buffer-B. Both cuvettes were placed in the dark then the optical density at 240 nm of the solution was recorded at 45 and 60 seconds starting from the time when the extract was added to the cuvettes, using a UV-vis Spectrophotometer (Model 6405, Jenway Ltd. Felsted, Dunmow, Essex, England). The difference in optical density between the 45 and 60 seconds readings was used to calculate CAT activity as reported by Luck (1965) and was expressed as enzyme unit activity mg^{-1} protein of skin or pulp. One unit CAT activity equaled the amount of enzyme that liberated half the peroxide oxygen from a H_2O_2 solution in the 100 s at 25°C.

Protein determination: Protein contents from fruit skin or pulp tissue were estimated using the standard method of Bradford (1976) and were expressed as mg g^{-1} FW.

Total antioxidants: The level of total antioxidants from fruit skin or pulp tissues were determined according to the method of Brand-Williams *et al*., (1995). Apple skin or pulp tissues (5 g) lyophilized with liquid nitrogen and stored at -80°C were homogenized in a glass pestle and mortar using 200 mg white quartz sand in 10 mL extraction buffer (2 mM NaF + 200 mL distilled water + 800 mL methanol). The homogenized tissue contents were centrifuged at 1000 x *g* for 15 min. Working solution of 2,2 diphynyl-1-picrylhydrazyl (DPPH) was prepared by diluting stock solution (24 mg DPPH + 100 mL methanol) with methanol (1:4, v/v) to 1.1 absorbance at 515 nm. The supernatant (50 µL) was mixed with DPPH working solution (950 µL) and the activity of total antioxidants was measured as the decrease in absorbance at 515 nm using UV/VIS Spectrophotometer (model 6405, Jenway Ltd. Felsted, Dunmow, Essex, England). Absorbance was measured

between 0.6-0.7 and the level of total anti-oxidant in the skin or pulp tissues were calculated against a standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) and were expressed as μM Trolox $100g^{-1}$ FW.

Statistical analysis: Data were analyzed by using one-way ANOVA using Genstat release 9.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effects of various treatments were assessed within ANOVA for various parameters and least significant differences (Fisher's LSD) were calculated following a significant F test ($p \leq 0.05$). All the assumptions of analysis were checked to ensure validity of statistical analysis.

Results

Changes in ethylene production and respiration rate during fruit maturation: During first experiment apple fruit harvested 7 d prior and up to 14 DACH did not show any significant change in the respiration rate. Later on 21 DACH apple fruit exhibited steady increase in respiration rate and reached at its maximum (0.97 mmol kg-1 h-1) on 28 DACH about 40% higher than fruit harvested at commercial maturity (Fig. 1A). No significant change in ethylene production was observed in harvested fruit up to 28 DACH then ethylene production increased and fruit exhibited climacteric ethylene peak (265 μmol kg-1 h-1) on 35 DACH (Fig. 1B).

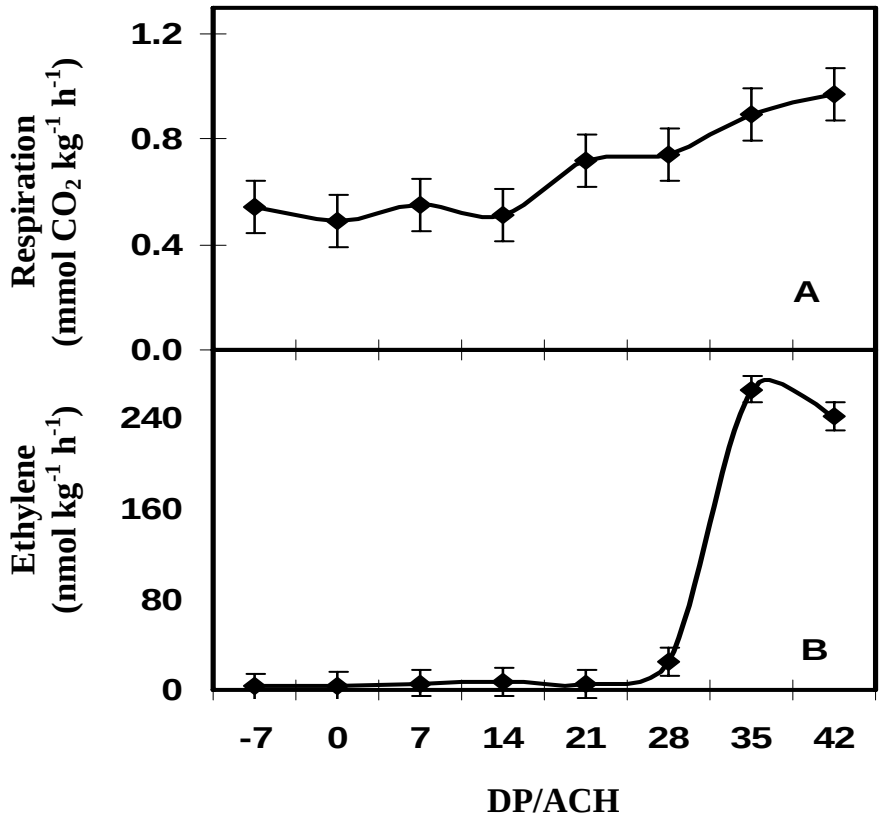

Fig. 1. Changes in respiration rate (A) and ethylene production (B) of fruit at different stages of maturity and ripening in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for respiration rate = 0.11 and ethylene production = 0.61. DP/ACH = Days prior/after commercial harvest. n = 18 (6 fruits x 3 replications).

Changes in fruit firmness, SSC, TA and SSC:TA ratio during fruit maturation and ripening: Apple fruit exhibited decrease in flesh firmness with increase in the harvest maturity. Fruit harvested 7 DPCH were 14.6% more firm, whilst fruit harvested 42 DACH were 16% more soft in contrast to fruit harvested at commercial maturity (Fig. 2A). Starting from 7 DPCH up to 28 DACH, apple fruit showed continuous increase in the SSC and SSC:TA ratio and later on no significant change occurred in SSC and SSC: TA ratio (Fig. 2B & 2D). Fruit TA decreased with increase in the fruit harvest maturity up to 35 DACH (Fig. 2C). In second experiment, fruit firmness and TA decreased, and fruit SSC and SSC:TA ratio increased with increase in fruit ripening period (Fig. 3).

Changes in the activities of SOD enzymes in fruit skin and pulp tissues during fruit maturation and ripening: With increase in fruit harvest maturity fruit skin tissue exhibited decrease in SOD activity up to 7 DACH then on 21 DACH skin tissue showed fast decline in SOD activity 36% lower than at commercial maturity (Fig. 4A). Later on fruit skin tissue exhibited sharp rise in SOD activity and it reached at maximum level (82 unit activity mg^{-1} protein) on 42 DACH. Pulp tissue also showed similar trend in SOD activity as was observed in skin tissue. SOD activity in pulp tissue decreased gradually up to 28 DACH and then increased SOD activity during last two harvest dates (Fig. 4B). Mean SOD activity during delayed fruit harvest period was 3.2 fold higher in skin pulp tissues. Fruit harvested at commercial maturity did not exhibit any significant change in the SOD activity in skin tissue up to 10 d of ripening period. Later on SOD activity in skin tissue increased and on day-20 skin tissue showed 32% higher SOD activity than in skin of fruit harvested at commercial maturity (Fig. 5A). During ripening period, pulp tissue exhibited 5% decline in SOD activity during first 5 d and later up to 20 d of fruit ripening pulp tissue showed 31% increase in SOD activity in contrast at commercial harvest (Fig. 5B). During fruit ripening, the mean SOD activity in skin tissue was 4.2 fold higher than pulp tissue.

Changes in the activities of CAT enzymes in fruit skin and pulp tissues during fruit maturation and ripening: Fruit skin tissue did not exhibit any significant change in CAT activity from 7 d prior commercial harvest. Later on skin tissues exhibited significant increase in CAT activity up to 21 DACH. Skin tissue on 28 and 35 DACH showed decline in CAT activity which again increased and highest CAT activity in skin tissue was observed on 42 DACH 68% higher than of fruit harvested at commercial maturity (Fig. 6A). Pulp tissue also showed similar trend in the activity of CAT enzymes, increased with increase in the fruit harvest maturity highest CAT activity was observed at 42 DACH (Fig. 6B). Mean CAT activity in skin tissue was 1.2 fold higher than pulp tissue. After commercial harvest skin tissue exhibited linear increase in activity of CAT enzymes (Fig. 7A). Skin tissue on day-15 of fruit ripening period showed highest SOD activity (111 unit activity mg^{-1} protein) 67% higher than at day-0 of fruit ripening period. Similar to fruit skin tissues, pulp tissue also exhibited similar trend in the increase of CAT activity. However, mean CAT activity in skin tissue was 2.14 fold higher than pulp tissue (Fig. 7B).

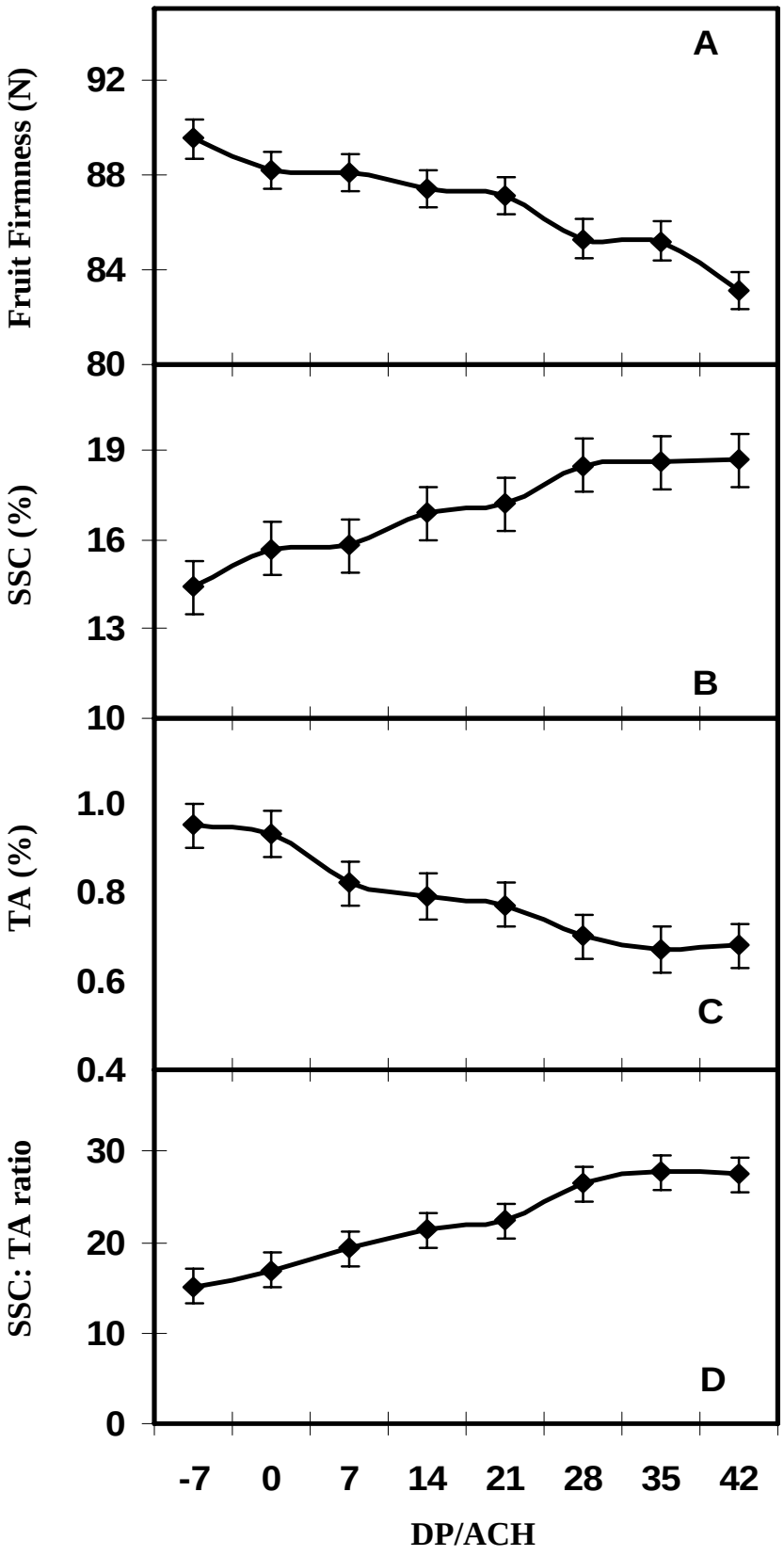

Fig. 2. Changes in the fruit firmness (A), soluble solid contents (SSC) (B), titratable acidity (TA)(C), and SSC:TA ratio (D) of fruit at different stages of maturity and ripening in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for firmness = 1.37. SSC = 0.87. TA = 0.03. SSC:TA ratio = 2.13. DP/ACH = Days prior/after commercial harvest. n = 18 (6 fruits x 3 replications).

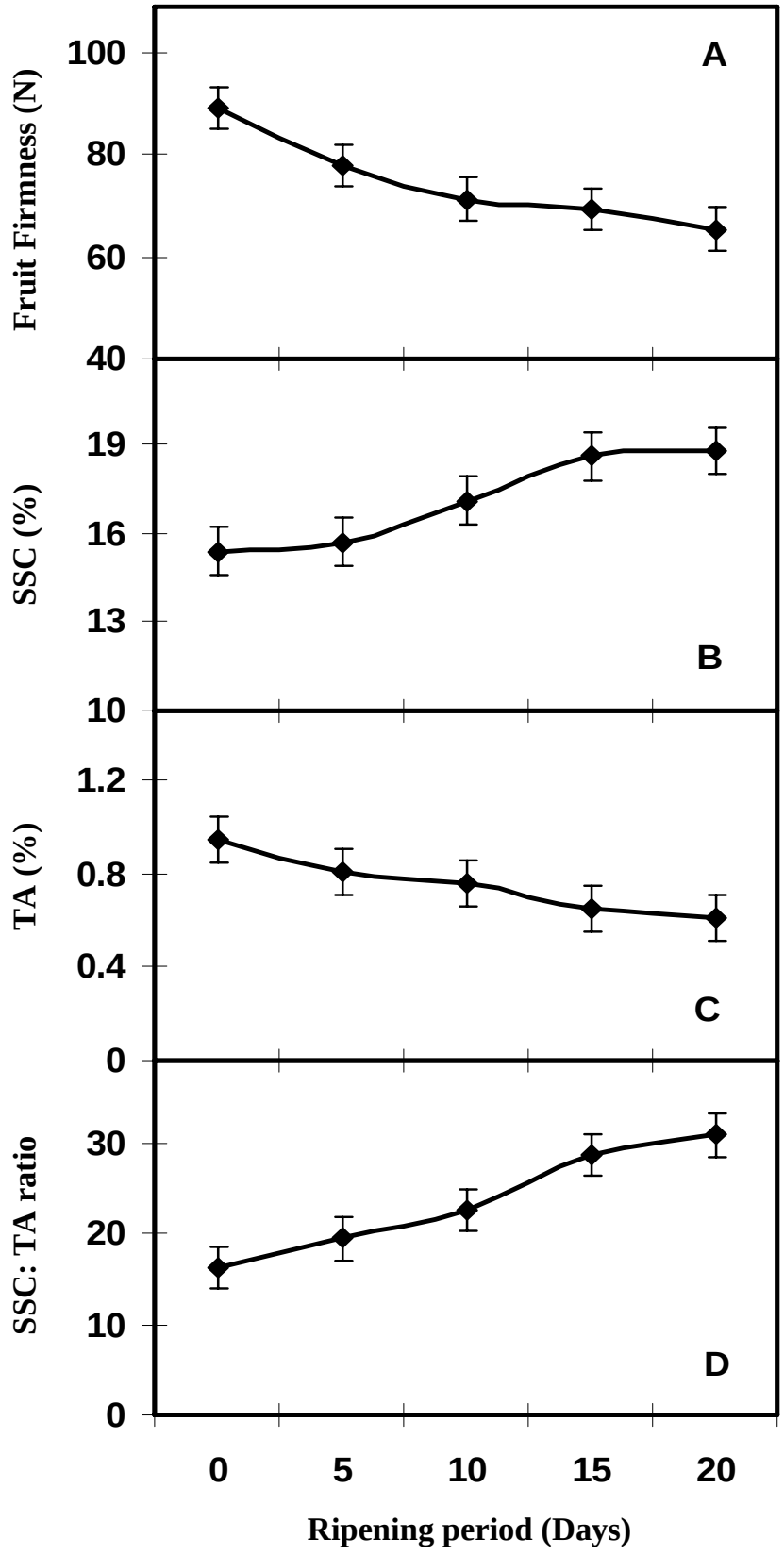

Fig. 3. Changes in the fruit firmness (A), soluble solid contents (SSC) (B), titratable acidity (TA)(C), and SSC:TA ratio (D) of fruit during ripening period at 22 ± 0.5°C in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for firmness = 1.21. SSC = 0.84. TA = 0.27. SSC: TA ratio = 2.11. DP/ACH = Days prior/after commercial harvest. n = 18 (6 fruits x 3 replications). n = 60 (20 fruits x 3 replications) for fruit firmness and n = 3 for SSC, TA and SSC: TA ratio.

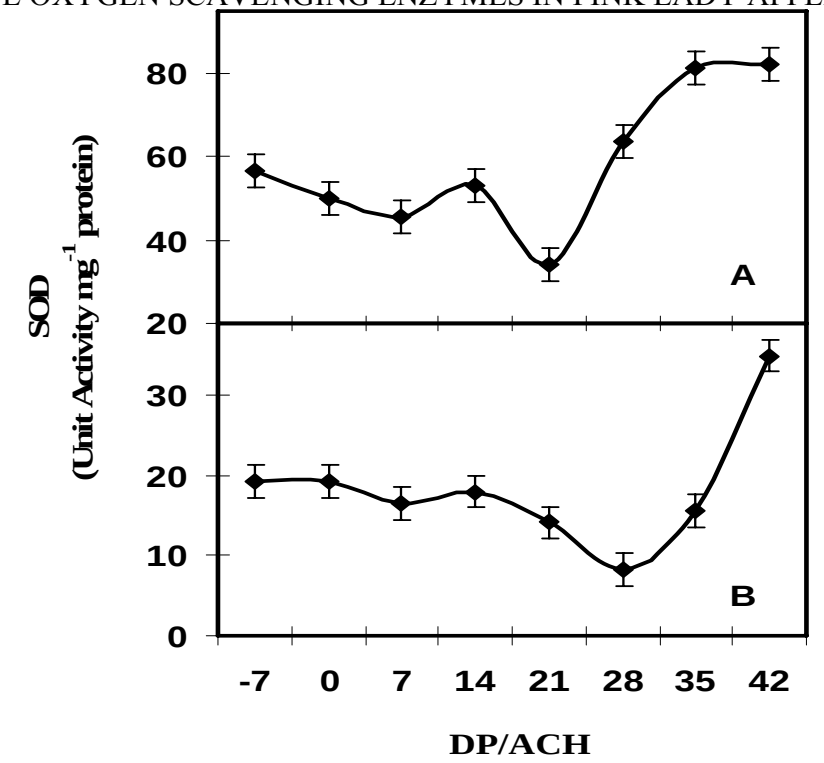

Fig. 4. Changes in the activity of SOD in skin (A) and pulp (B) of fruit at different stages of maturity and ripening in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for skin = 3.9 and pulp = 2 DP/ACH = Days prior/after commercial harvest. n = 18 (6 fruits x 3 replications). n = 3 (3 replicates).

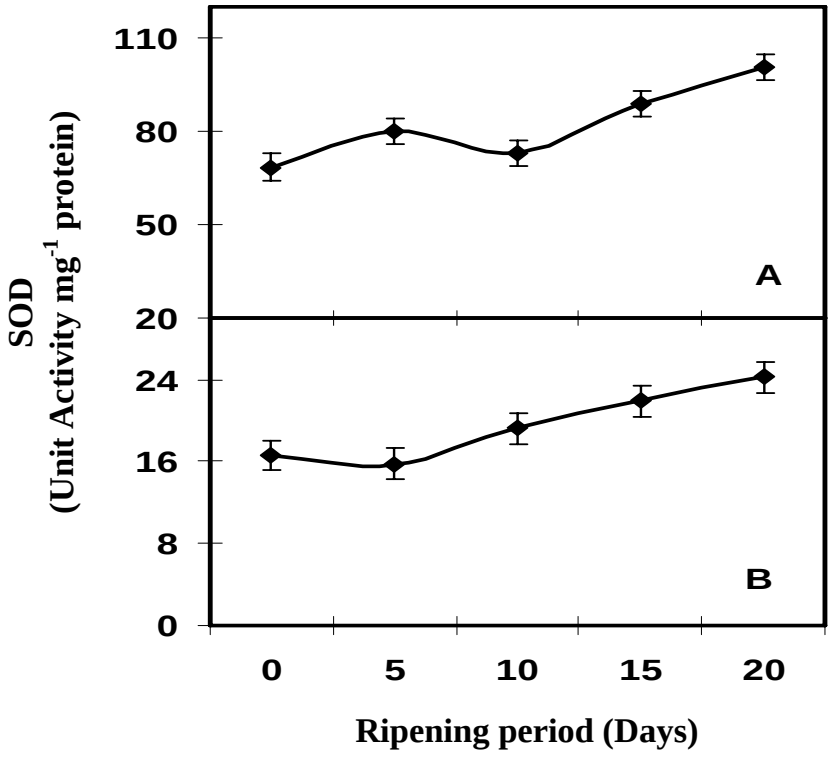

Fig. 5. Changes in activity of SOD in skin (A) and pulp (B) of fruit during ripening period at 22 ± 0.5°C in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for skin = 10.3 and pulp = 1.7. n = 3 (3 replicates)

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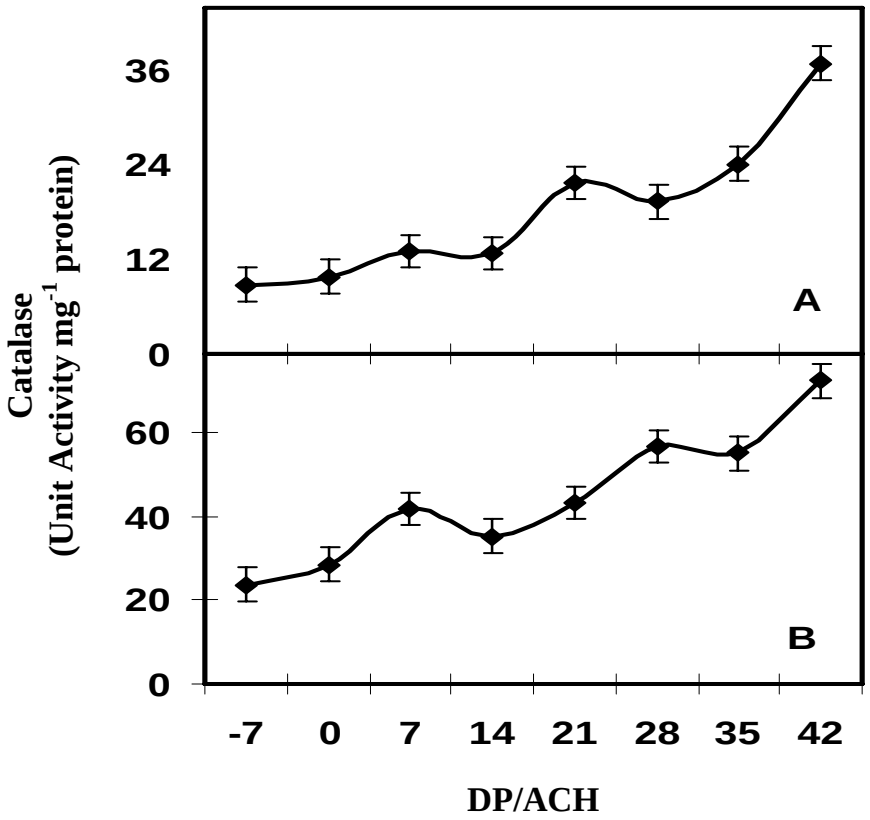

Fig. 6. Changes in activity of CAT in skin (A) and pulp (B) of fruit at different stages of fruit maturity and ripening in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for skin = 8.9 and pulp = 2.8. DP/ACH = Days prior/after commercial harvest. n = 3 (3 replicates).

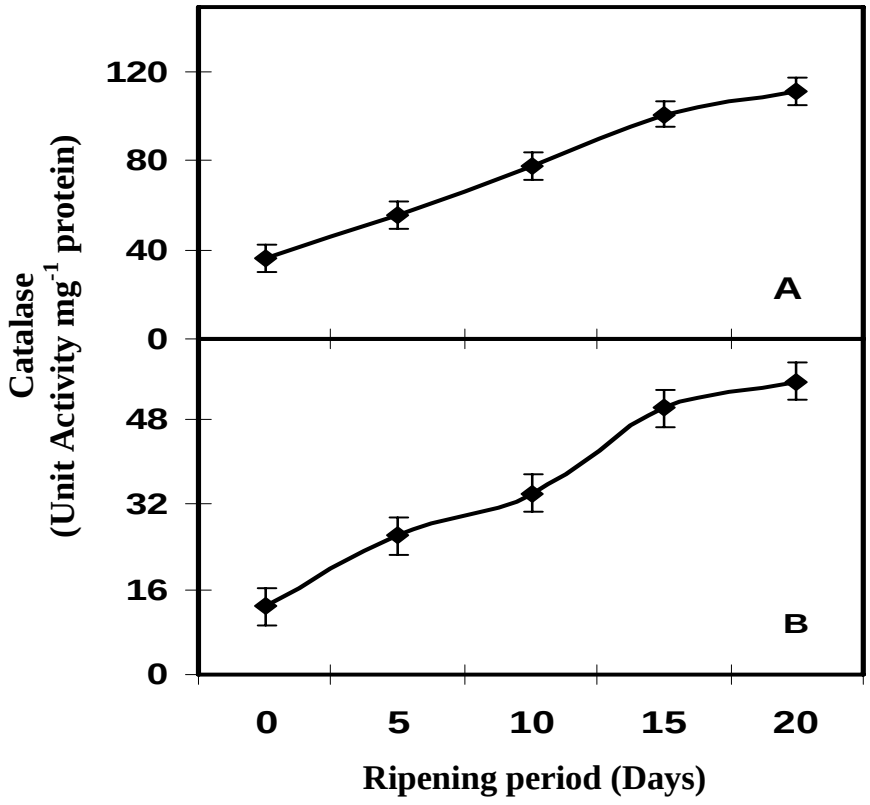

Fig. 7. Changes in the activity of CAT in skin (A) and pulp (B) of fruit during ripening period at 22 ± 0.5°C in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for skin = 8.9 and pulp = 2.9. n = 3 (3 replicates).

Changes in the levels of total antioxidants in fruit skin and pulp tissues during fruit maturation and ripening: Seven days prior to commercial harvest and during delayed harvest, skin tissue did not show any significant change in the level of total antioxidants with the exception of 21 DACH skin tissue, which exhibited 13% decline of total antioxidants compared to commercial harvest (Fig. 8A). Pulp tissue showed a slight decrease in the level of total antioxidants from 7 d prior to commercial harvest to commercial harvest. Later on pulp tissue exhibited a gradual increase in level of total antioxidants up to 21 DACH. Pulp tissue on 28 DACH exhibited 6% reduction in the level of total antioxidants than in pulp of fruit harvested at commercial maturity. During the last two harvests, the level of total antioxidants in pulp tissue increased and reached the maximum level (1.5 µM Trolox $100g^{-1}$ FW) at 42 DACH (Fig. 8B). Skin tissue exhibited a 1.4 fold higher mean level of total antioxidants as compared to pulp tissue. Skin and pulp tissues of fruit harvested at commercial maturity did not exhibit any significant change in the level of total antioxidants during fruit ripening period (Fig. 9A & 9B). However, the mean level of total antioxidants during fruit ripening period in skin tissue was 1.34 folds higher than pulp tissue.

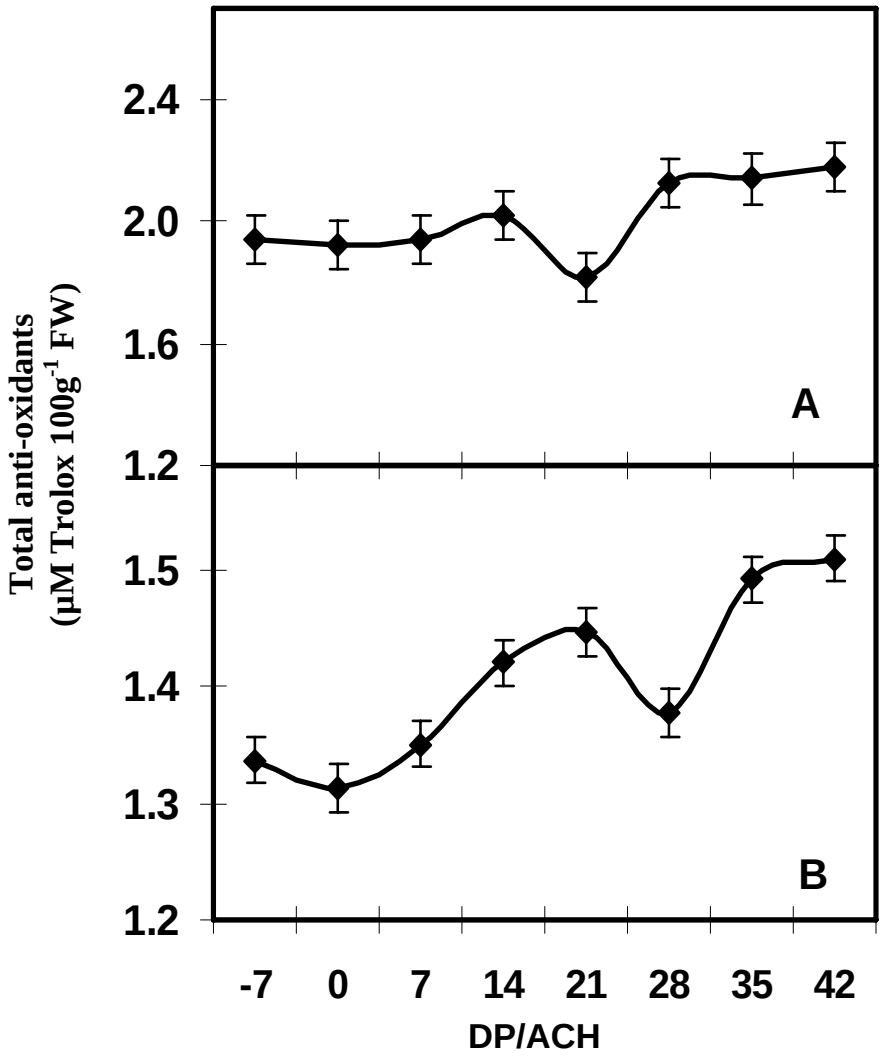

Fig. 8. Changes in total antioxidants in skin (A) and pulp (B) at different stages of fruit maturity during harvest period in 'Pink Lady' apple. Vertical bars represent ± SE of mean. LSDs ($p \leq 0.05$) for skin = 0.325 and pulp = 0.090. DP/ACH = Days prior/after commercial harvest. n = 3 (3 replicates).

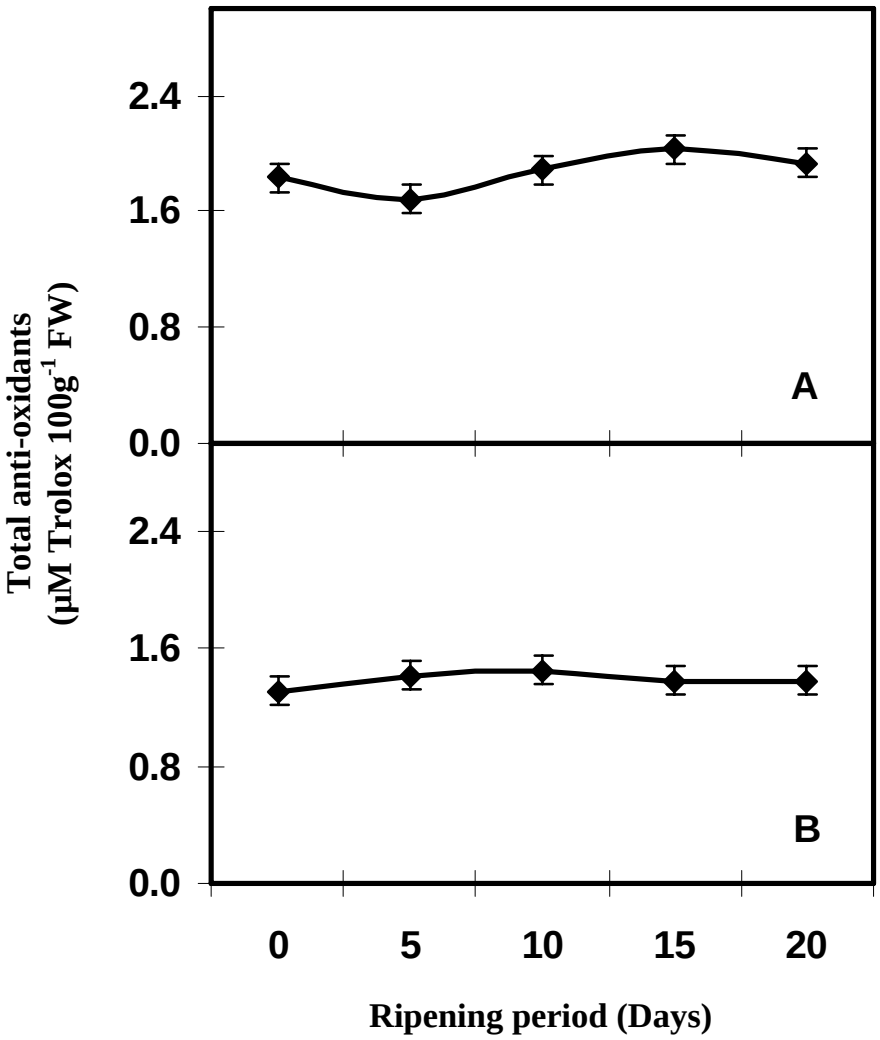

Fig. 9. Total antioxidants in skin (A) and Pulp (B) of fruit during ripening period at 22 ± 0.5°C in 'Pink Lady' apple. Vertical bars represent ± SE of means. LSDs ($p \leq 0.05$) for skin = 0.343 and pulp = 0.228. n = 3 (3 replicates).

Discussion

In present study fruit were harvested at the pre-climacteric stage, 7 d prior to commercial harvest, and respiration rate and ethylene production increased with advancement of fruit maturity (Fig. 1). Fruit exhibited the climacteric phase on 35 DACH with autocatalytic rise in ethylene production. This increase in respiration rate and ethylene production might be due to climacteric nature of apple fruit. Similarly, rise in ethylene production and respiration rate has been reported in 'Red Delicious' apple fruit during different harvest periods (MacLean *et al*., 2006). Recently, higher levels of endogenous ethylene has also been observed in 'Pink Lady apple with advancement in fruit maturity (Whale & Singh, 2007). Fruit firmness progressively declined throughout the extended period fruit harvest maturity and ripening (Fig. 2A & 3A). The decline in fruit firmness during fruit harvest maturity and ripening can be attributed to the hydrolysis of cell walls by the action of enzymes like polygalacturonase and pectin methylesterase enzymes (Fischer & Bennett, 1991). SSC and SSC:TA of the fruit progressively increased whilst TA decreased both during advanced fruit maturity and ripening (Fig. 2 & 3), which can be attributed to the hydrolysis of starch to sugar (Akhtar

et al., 2010). Fructose, glucose and sucrose are the main sugars that accumulate during apple fruit maturity and subsequent ripening (Beruter, 2004). Reduction in the TA is due to decarboxylation of malate by malic acid enzyme and the consequent decarboxylation of private (Hawker, 1969).

Oxidative stress is involved in many biological systems, among which are fruit ripening and senescence (Masia, 1998). In the present study the fruit were kept on-tree for extended period of maturity and later on for ripening after commercial maturity, so that fruit must expose to some degree of oxidative stress. The level of SOD activities in fruit skin and pulp tissues first decreased up to 28 DACH and then increased on 28 DACH corresponding with an increase in the respiration rate and ethylene production (Fig. 1A). Earlier, it has been reported that the onset of ripening was associated with activities of SOD and CAT in cantaloupe melons (Ben-Amor *et al*., 1999). Earlier delayed harvest resulted in a similar changes in SOD and CAT activities in pear (Lentheric *et al*., 1999) and SOD activity in 'Braeburn' apple fruit (Gong *et al*., 2001). The decline in scavenging capacity along with increased respiration might have resulted in the accumulation of ROS which then triggered the activity of anti-oxidative enzymes and ethylene biosynthesis that became more obvious in the fruit during last two harvests in this study. Ethylene production followed the same pattern as that of SOD activity. This rise in SOD activity might be signaling the initiation of tissue senescence as was observed in previous findings (Du & Bramlage, 1994). However, SOD activity was found to be strongly cultivar specific and has shown different patterns of sensitivity to stress in different apple cultivars. Contrarily, peaks of SOD and CAT activities were corresponded with climacteric ethylene peak in 'Golden Delicious' and 'Fuji' apples (Masia, 1998). Increased levels of superoxide radicals are also associated with increase in SOD activity in tomato (Barka, 2001). Senescing tissues high in oxy radicals were found to have increased SOD activities in apple fruit (Du & Bramlage, 1994).

Apple fruit harvested at commercial fruit maturity, and ripened at 22^{o}C have shown an overall increase in the SOD activity in the peel after an initial drop up to 10 days. However, in general SOD activity in the pulp declined over the ripening period (Fig. 3A & 3B). As discussed above that onset of ripening might be associated with decline in SOD activity in fruit. The fruit might have reached to the climacteric peak within 10 d of ripening period, which resulted in the decline of SOD activity. In the same manner as in on-tree ripening, the fruit recovered the SOD activity after the initiation of ripening may be in response to oxy radicals generated as a result of decompartmentalisation. Du, & Bramlage (1994) examined sharp decline in SOD activity with the onset of ripening in 'Empire' apples. It is apparent form our experimental data that pulp show very low SOD activity and is not much responsive to physiological changes compared to the skin of the fruit.

Results of the present study revealed that CAT activity increased in the skin of apple fruit in a continuous manner with enhancement in fruit maturity reaching to the highest level on 42 DACH (Fig. 6A). In pulp the activity changed in almost the same manner as in skin. However, gradual increase in CAT activity is coincided with the advancement of fruit maturation and ripening (Fig. 6B). CAT is of paramount importance in detoxification of H_2O_2. It acts on H_2O_2 at lower amount than other enzymes (Barka, 2001). H_2O_2 is capable of rapid diffusion across the cell membranes and may act as second messenger in selective induction of defence genes (Foyer *et al*., 1997). In ripening fruit, the rise in H_2O_2 and other reactive oxygen species is accompanied by a parallel rise in the ethylene concentration (Leshem *et al*., 1986a; Masia, 1998). The rise in CAT activity during enhanced fruit maturity in present study might be stimulated by the increased level of

H_2O_2 production as a result of climacteric peak. A significant increase in the activity of CAT was observed in the skin and pulp of 'Pink Lady' fruit with advancement in ripening (Fig. 7). Previous studies have shown a strong relation between surge of cellular oxidants at the onset of ripening and increase in anti-oxidant enzymes activities including CAT (Masia, 1998). The increase in CAT activity in present study suggests a continuous production of ROS during fruit ripening period at high temperature (22^{o}C) with a parallel increase in their detoxifying enzymes activities. Level of total antioxidants in skin did not show any significant change during enhanced fruit maturity except on 28 DACH at which the level of total antioxidants reduced (Fig. 8A). However, pulp tissue exhibited increased level of total antioxidants with advancement of fruit maturity up to 21 DACH and later it increased again on tree fruit ripening (Fig. 8B). Skin showed 30% higher level of total antioxidants than pulp tissue as reported earlier in 'Rome Beauty', 'Idared', 'Cortland' and 'Golden Delicious' apples (Wolfe *et al*., 2003).

In conclusion 'Pink Lady' apple fruit can with stand anti-oxidative stress at advanced stages of fruit harvest maturity (up to 42 DACH) and during subsequent fruit ripening (up to 20 d) with sufficient levels activities of SOD and CAT enzymes as well as total antioxidants.

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