

**EFFECT OF CONTROLLED ATMOSPHERE STORAGE (CAS) ON
ANTIOXIDANT ENZYMES AND DPPH- RADICAL SCAVENGING
ACTIVITY OF MANGO (*MANGIFERA INDICA* L.) CV. ALPHONSO**

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ABSTRACT

Alphonso mangoes (*Mangifera indica L.*) pre-treated with either N-propyl-N-[2-(2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide (prochloraz) or hot water (HW) were stored under controlled atmosphere (CA) consisting of 5% O₂ and 5% CO₂ for 45 days at 8°C followed by ripening at ambient conditions. Pre-treatments had a pronounced effect on storage life as well as disease incidence. Untreated fruits stored in CA were completely rotten. Irrespective of pre-treatments fruits stored in air showed chilling injury (CI) symptoms during storage at 8°C and intensity further increased during ripening. Prochloraz or HW pre-treatment followed by CA storage resulted in fruits absolutely free from morphological CI with fresh appearance, hard, green and ripened normally when shifted to ambient conditions (24-29° C, 60-70% RH). Effect of pre-treatments and CA storage on antioxidant enzymes viz. catalase (CAT) peroxidase (POX) and superoxide dismutase (SOD) was compared. The unripe air stored fruits (untreated, prochloraz treated and HW-treated) after 45 days of storage at 8° C showed significantly higher CAT and SOD activity and lower POX activity in comparison to that stored in CA (prochloraz and hot water treated fruits). Freshly harvested matured green fruits showed comparatively less CAT activity than pretreated and stored fruits. Unripe, prochloraz and HW-pretreated fruits of CA storage and freshly harvested fruits have shown similar SOD activity. After 45 days of CA storage at 8°C, POX activity in prochloraz and HW-treated fruits was higher than that found in freshly harvested fruits. After ripening under ambient conditions, prochloraz and HW-treated fruits stored in both CA and air showed significantly higher antioxidant enzyme (CAT, POX and SOD) activities than corresponding untreated fruits. Total phenol content and 2, 2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of fruits decreased during CA storage and after ripening when compared to freshly harvested fruits. Decrease was also significant between unripe and ripened fruits of different treatments. Prochloraz or HW-treated fruits stored in CA had highest carotenoids content compared to fruits stored in air after they were ripened at ambient conditions. The present study revealed that CA storage helped in retaining the antioxidant levels of Alphonso mangoes when they were pre-treated with prochloraz or hot water.

Key words: Antioxidants, Prochloraz, Catalase, Peroxidase, Mango

INTRODUCTION

Fruits after harvest and before consumption, encounter several stress factors simultaneously like heat shock or storage at low temperature. Under optimum conditions, cellular homeostasis has been achieved by a coordinated action of several biochemical pathways. Stress factor may affect biochemical pathways. Reactive oxygen species (ROS) have been continuously produced due to various metabolic activities and production increases under stress conditions along with the activation of various defense genes, which may include ROS scavenging, and stress proteins [1]. Protection from such damages could be characterized by activation and deactivation of antioxidant defense enzymes, such as Superoxide Dismutase (SOD), Peroxidase (POX), Catalase (CAT), Ascorbate peroxidase (APX) and Glutathione Reductase (GR) and also by natural antioxidants, such as Ascorbic acid, Beta carotene, and Glutathione [2].

Mango is an important tropical fruit known for its characteristic aroma and taste. Mango has limited shelf life when held at low temperature due to chilling injury. Researchers have attempted to study several treatment methods to increase the shelf life of mango include low temperature storage, controlled atmosphere and modified atmosphere or combination of both [3]. Pre-storage treatments, intermittent warming, temperature conditioning and nitrogen gas exposure and also stage of harvest have been reported to reduce chilling injury [4]. Mangoes were reported to ripen satisfactorily with acceptable eating quality between 21 and 24°C. A low temperature of 12-13°C is generally considered as optimum for storage of mango. Storage of mangoes below 10°C resulted in chilling injury (CI) manifested in grayish scald like discoloration of the skin, skin pitting, uneven ripening, reduction in the levels of carotenoids, aroma and flavor during ripening and susceptibility to fungal decay [5]. Modified atmosphere/controlled atmosphere storage of fresh horticultural crops using 2 to 3% O₂ and < 5% CO₂ effectively reduce or inhibit C₂H₂ induced senescence, reduces respiratory metabolism, maintains flesh firmness and color and controls physiological disorders in harvested fruits and vegetables. Even though several workers have attempted to evaluate the beneficial and detrimental effects of controlled and modified atmosphere on fresh fruits and vegetables, the mode of action of Oxygen and CO₂ on these commodities still remains to be understood [6]. Changes in carotenoids, ascorbic acid, carbohydrates, TSS, acid levels and other quality attributes during ambient storage/low temperature storage have been well studied whereas reports on the antioxidant enzymes and antioxidants on CA stored Alphonso mango have been found to be very meager and thus were considered for the present study.

MATERIALS AND METHODS

Fruit Material

Mature fruits were selected for experiment. Fruits were sorted out for mechanical injuries like abrasions, punctures, and bruises and graded as per size, shape and colour to maintain uniformity in the experiment. These fruits were washed in running water to remove the adhering latex, dust, and dirt. Excess moisture was allowed to drain off. The mango fruits were divided into three groups and each group contained 60 fruits.

Fungicide treatment

Group I: The fruits were dipped in 500 ppm of prochloraz [45%ECTM] fungicide [Indofil Chemicals Co., Thane] emulsions for 10 minutes. Treated fruits were taken out and excess fungicide solution was drained off. Fruits were dried to remove the excess surface moisture.

Hot water treatment

Group II: Fruits were dipped in hot water tank (maintained at 55°C) for 5 minutes.

Group III: Fruits were immersed in tap water tank followed by drying to remove the excess surface moisture.

Pre-cooling

Prochloraz treated, HW-treated and untreated fruits were pre-cooled to 8°C by using forced air pre-cooler to remove fruit heat rapidly.

A thermometer was used to monitor fruit temperature. Fruits of each treatment were divided equally into two sub-groups. Thirty fruits of each sub group were stored in air at 8°C as control of the pre-treated fruits (untreated stored at air, prochloraz treated stored at air and HW-treated stored at air) whereas other subgroups of each pretreated were subjected to CA storage in the storage chambers at 8°C (untreated CA storage, prochloraz treated CA storage and HW-treated CA storage).

Controlled Atmosphere storage

Controlled Atmosphere storage chambers were calibrated to establish the specified gas composition – 5% O₂, 5% CO₂ and 90% N₂ by a gas blending flow system. The gas blending system generated CA conditions using external supplies of gases from pressurized gas cylinders fitted with double-stage regulators and outlet controlling devices. Gas (O₂, CO₂ and N₂) flows were restricted through needle-valves. The system was designed in such a way that four different gas combinations could be achieved at any time, with four outlets each passing through gas flow meters wherein the final outlet flow of blended gases could be precisely controlled. These outlets were connected to the inlet flexible (Tygon®) pipes that were inserted into the desiccators in which the mangoes were stored. The CA system was a continuous gas flow, open-ended system without humidification. To achieve uniformity in the final flow, the outlet pressure from the gas cylinders was reduced step-by-step using sensitive pressure regulator valves and pressure indicating dials. The inlet flow rate per each desiccator containing 30 mangoes was set at 30 ml min⁻¹ with a turn over rate of 7.77.

Extraction of antioxidant enzymes

Five grams of frozen mango pulp was ground in liquid nitrogen using mortar and pestle chilled with liquid nitrogen and their fine powder was used for extraction of antioxidant enzymes. Super oxide dismutase (SOD) and CAT enzymes were extracted by following the protocol described by Bailly *et al.* [7] and POX was extracted using the protocol of Hanotel *et al.* [8].

Assay of Antioxidant enzymes

Superoxide dismutase activity was assayed by measuring the ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) [9]. Enzyme activity was measured spectrophotometrically at 560 nm. One unit of SOD was considered to be the amount of enzyme that inhibited NBT reduction by 50%.

The decomposition of H₂O₂ by CAT was measured spectrophotometrically at 240 nm. One unit of CAT activity corresponded to the amount of enzyme that decomposes one μM of H₂O₂ per minute [10].

The POX activity was measured spectrophotometrically at 485 nm in reaction mixture consisting of 0.1 M potassium phosphate buffer (pH 7) 12.5 mM o-phenylenediamine and 5 mM H₂O₂ [11]. One unit of POX activity corresponded to the amount of enzyme responsible for change in absorbance of 1 absorbance unit min⁻¹. All enzyme activities were expressed per g of fresh weight (FW).

Estimation of Total Phenol and 2, 2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Total phenolic content of the fruit pulp was determined by following the method of Singleton and Rossi [12]. Absorbance was read at 725 nm. The DPPH radical scavenging activity of the phenol extract of fruit pulp was estimated according to the method of Lai *et al* [13]. Absorbance was recorded at 517 nm. IC₅₀ value was determined from the plotted graph of scavenging activity against the total phenol concentration of the fruit pulp extract and expressed in terms of IC₅₀ (IC₅₀ concentration in μg of gallic acid equivalents required for a 50% decrease in absorbance of DPPH radical).

Estimation of carotenoids

Carotenoids were estimated by following standard AOAC method [14]. Optical density values (OD) were recorded using spectrophotometer at 450 nm. Carotene content was calculated with reference to the standard curve prepared with β-carotene. The results were expressed as β-carotene equivalents 100⁻¹ g of pulp.

Statistical analysis

Data generated was subjected to one-way ANOVA and means were separated using the CD values at 5% and depicted in the Figures and Tables.

RESULTS

Storing of fruits or vegetables in controlled atmosphere (CA) enriched with high CO₂ and/or utilizing low O₂ levels could be a very beneficial tool to maintain quality of the produce. In the present investigation, combination of low O₂ (5%) and high CO₂ (5%) concentration of gases were used in CA storage and extended the storage life of Alphonso mango (45 days) at 8°C. Pre-treatments - prochloraz and HW had prevented disease incidence whereas untreated fruits stored in CA were prone to diseases. Pretreated CA stored fruits were absolutely free from morphological CI and fruits

appeared fresh, hard and green after 45 days storage at 8°C. Irrespective of the treatments (untreated, prochloraz treated and HW-treated), fruits kept in air developed dark scald-like discolorations in the peel which began around lenticels and spread outwards, produced circular lesion and pitting on the fruit peel. CI symptoms were further increased when these air-stored fruits were shifted to ambient temperature for ripening. Prochloraz and HW-treated fruits stored in CA were free from CI and ripened normally when shifted to ambient conditions (24-29°C, 60-70% RH).

Effects of CA storage on Antioxidant enzymes

Catalase (CAT) activity

Effect of pre-treatments and CA on CAT was compared (Fig 1). Pre-treatments and CA storage had significant impact on CAT activity ($5.98 \mu\text{M H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}\text{FW}$) in comparison to freshly harvested mango fruit. Control fruits (untreated, prochloraz and HW-pretreated) stored at air showed significantly higher CAT activity ($63.0, 59.6, 62.4 \mu\text{M H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}\text{FW}$ respectively) than prochloraz and HW-treated fruits stored in CA. Hot water treated CA stored fruits had higher CAT activity ($57.4 \mu\text{M H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}\text{FW}$) in comparison to prochloraz pretreated CA stored fruits ($44.2 \mu\text{M H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}\text{FW}$). CAT activity was not measured in untreated CA stored fruits because all fruits were spoiled.

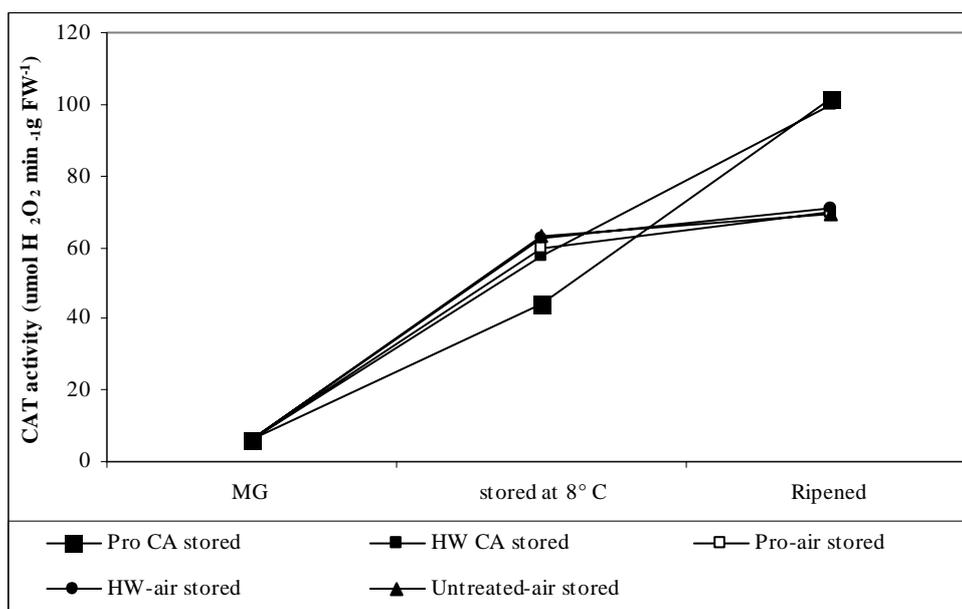


Figure 1: CAT activity during CAS and post CA ripening.
 (CAT – SEM = 1.9, C.V. (%) = 6.65 and CD at 5% = 5.44)

During post CA ripening, CAT activity in prochloraz and hot water pretreated CA stored fruits was 101.4 and 100 ($\mu\text{M H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}\text{FW}$) respectively. Similarly, control fruits, hot water treated, prochloraz treated and untreated stored in air at 8°C followed by ripening at ambient conditions has shown CAT activity as 70.8, 70.0 and 69.2 units ($\mu\text{M H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}\text{FW}$), respectively.

Peroxidase (POX) activity

Peroxidase activity in fruits of freshly harvested, untreated, prochloraz and HW-pretreated varied much less (0.51, 0.50, 0.48 and 0.52 unit's min⁻¹ g⁻¹FW, respectively). Controlled atmosphere storage had a significant impact on POX activity indicated by 1.22 units min⁻¹ g⁻¹FW in prochloraz pre-treated CA stored and 1.38 units min⁻¹ g⁻¹FW in HW-pretreated CA stored fruits (Fig 2). Even during post CA ripening, similar response of CA stored was observed in the POX activity of prochloraz and HW-pretreated fruits (1.50 and 1.44 units min⁻¹ g⁻¹FW POX activity respectively). Increase in POX activity was also found in untreated, HW-treated and prochloraz treated fruits (0.80, 0.94 and 1.00 units min⁻¹ g⁻¹FW, respectively) stored in air.

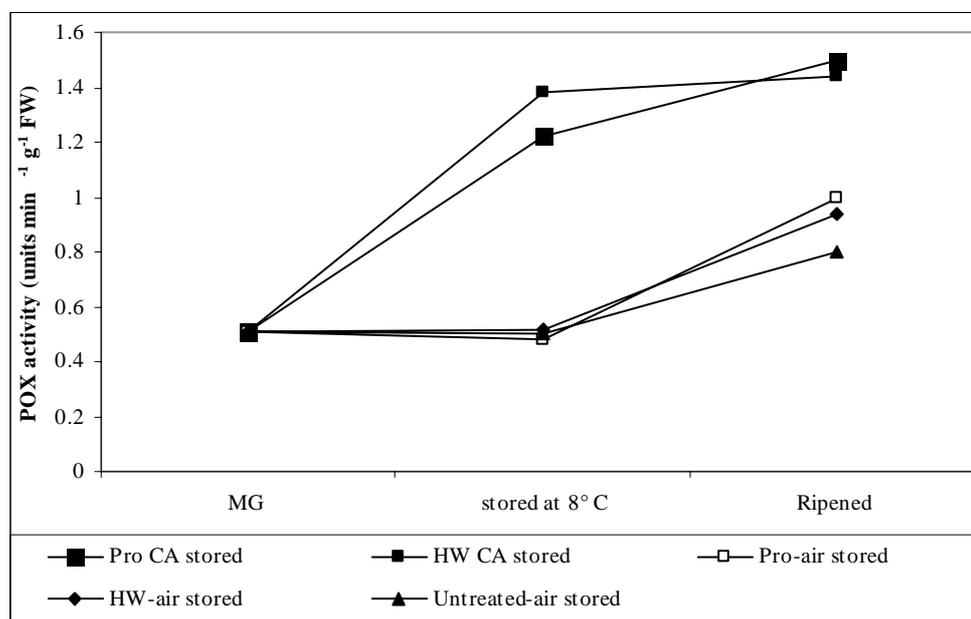


Figure 2: POX activity during CAS and post CA ripening.
 (POX - SEM = 0.1, C.V. (%) = 24.9 and CD at 5% = 0.3)

Superoxide dismutase activity

Changes in SOD activity was measured (units min⁻¹ g⁻¹ FW) in CA stored and air stored fruits for period of 45 days at 8°C and followed by ripening at ambient conditions (Figure 3). Superoxide dismutase activity in freshly harvested fruits at harvest, prochloraz pre-treated CA stored and HW-treated CA stored was more or less the same (119.4, 119.4 and 118.4 units min⁻¹ g⁻¹FW, respectively) whereas slight increase in the activity was observed in the control fruits stored at air (untreated, prochloraz and hot water pretreated as 124.2, 123.2 and 122.6 units min⁻¹ g⁻¹FW respectively). After ripening at ambient conditions air- stored fruits of untreated, prochloraz and HW-pretreated had shown decreased SOD activity (66.2, 67.6 and 67.0 units min⁻¹ g⁻¹FW, respectively). Fruits stored at CA (Prochloraz treated and hot water treated) conditions followed by ripening at ambient conditions had 97.0 and 98.0 units min⁻¹ g⁻¹FW SOD activity, respectively.

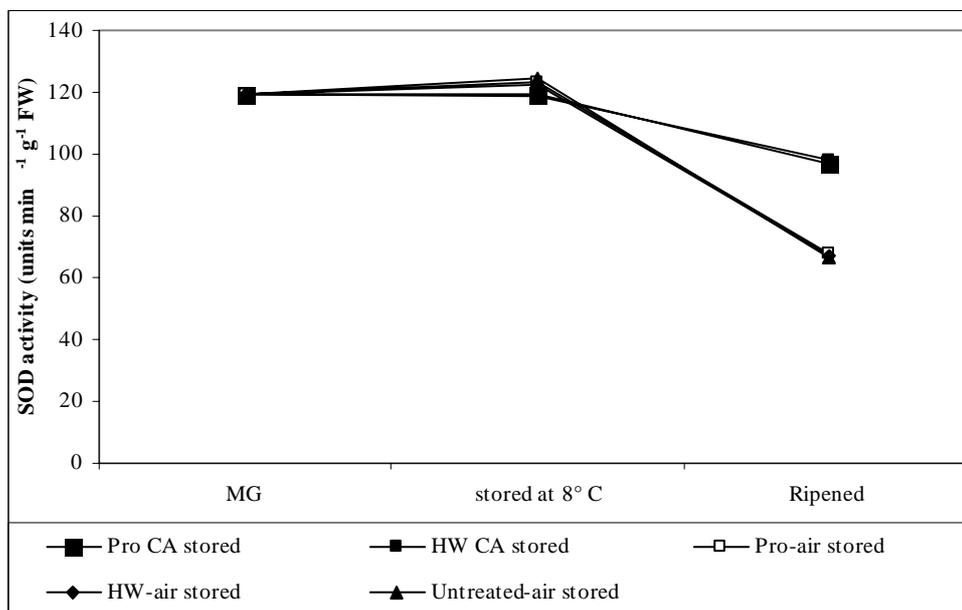


Figure 3: SOD activity during CAS and post CA ripening.
 (SOD - SEM = 1.45, C.V. (%) = 3.17 and CD at 5% = 4.14)

Effect of CAS on total phenols and DPPH radical scavenging activity

Total phenol

Table 1 indicates the variation in total phenol content as affected by CA storage and pre-treatments (Prochloraz treated and HW-treated). In fact, the total phenol content decreased in CA stored and post CA ripened fruits in comparison to freshly harvested fruits. Decreased total phenol content was recorded in fruits of HW-treated control (74.4 mg 100g⁻¹ FW) untreated control (75 mg 100g⁻¹ FW), prochloraz treated control (76.6 mg 100g⁻¹ FW) fruits stored in air, HW-treated (86.2 mg 100g⁻¹ FW) and prochloraz treated (86.6 mg 100g⁻¹ FW) fruits stored in CA. Control atmosphere stored and control fruits ripened at ambient conditions have shown further decrease in the total phenol content. The CA stored fruits retained significantly higher total phenol content than that stored in air. After ripening, estimated total phenol content in prochloraz treated CA stored, HW-treated CA stored, prochloraz treated control, HW-treated control and untreated control fruits was 60.8 58.6, 38.2, 36.0 and 36.6 mg 100g⁻¹ FW, respectively.

2, 2-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging capacity of CA stored and post CA ripened fruits has been expressed in terms of IC₅₀ (concentration in µg of gallic acid equivalents required for a 50% decrease in absorbance of DPPH radical) (Table 1). The DPPH radical scavenging capacity of mango pulp has shown a significant difference with the storage factors (CA stored and stored in air). In general, radical (DPPH) scavenging capacity of fruit pulp decreased during storage and ripening. Prochloraz treated CA stored, (5.96) and HW-treated CA stored (6.12) fruits were observed to be better radical scavengers than prochloraz treated control (7.64), HW-treated control (7.34)

and untreated control (7.42) fruits. Fruits (CA stored and control fruits stored in air) ripened at ambient conditions showed further decrease in DPPH radical scavenging capacity. Post CA ripened fruits were found to be better radical scavengers than control ripened fruits. Fruits of prochloraz treated CA stored, HW-treated CA stored, prochloraz treated control, HW-treated control and untreated CA control ripened at ambient conditions recorded 9.12, 9.74, 12.20, 12.28 and 12.50, respectively.

Carotenoids

There was no significant difference in carotene content of mangoes after 45 days storage in either CA or air (Table 1). Carotenoid content of post CA stored ripened fruits were significantly higher than control fruits. The type of CA storage and pretreatment (prochloraz or HW) did not have any effect. Prochloraz treated CA stored and its control, HW-treated and its control fruits, and untreated control fruit recorded increased carotene content of 6.7 and 5.38, 6.86 and 5.46 and 5.16 mg 100 g⁻¹ FW, respectively.

DISCUSSION

Controlled atmosphere regimes were beneficial in extending storage life of fruits and reducing the chilling injuries during storage and their ripening. Effectiveness of chilling injury reduction maintaining produce quality and extension of storage life in CA storage often depends on the type of commodity, concentrations of O₂ and CO₂, temperature and duration of storage [15]. In the present investigation, combination of low O₂ (5%) and high CO₂ (5%) concentration of gases used in CA storage had extended storage life of mango to 45 days and Noomhorm and Tiasuwan in their study with Rad mango have also reported the extension of storage life for 25 days at 13°C [16]. The CA stored fruits were absolutely free from CI injuries. Fruits kept in air developed visible CI. In the present CA experiments, selected pre-treatments have given effective control of the disease incidence. However, untreated CA stored fruits were completely rotten mainly due to anthracnose disease. Similar response was recorded while studying effect of hot water treatment and CA storage on Tommy Atkins mango [17].

Controlled atmosphere storage has been shown to affect the respiration, ethylene production and its action, enzyme synthesis and activity, ripening process and senescence of various horticultural products [6]. Results of the present investigation also revealed similar inference of the effect of CA storage on antioxidant enzymes and antioxidants. Catalase activity in the CA stored fruits was significantly increased in ripened fruits than in unripened. In unripe condition, control fruits stored in air showed significantly higher CAT activity than CA stored fruits that could be due to CI induced oxidative stress as also reported by Foyer and Harbinson [18]. Among the pre-treatments, HW-treated CA stored fruits induced a highly significant increase in activity compared to prochloraz treatments. Increased CAT activity in HW-CA stored fruits and its corresponding control fruits might be due to temperature-induced expression of CAT. Similarly, prochloraz treated CA stored and its corresponding air stored fruits have shown significantly lesser CAT activity than untreated control fruits. This may be due to hypersensitive response from infected untreated control

fruits infected by anthracnose and stem end rot since these spoiled fruits produced more ethylene. Maria *et al* [19] also reported that externally applied ethylene during cold storage of citrus fruits induced the H₂O₂ detoxifying enzymes. Prochloraz pre-treatment completely controlled the infection resulting in significantly least CAT activity.

Hydrogen peroxide is known to be detoxified by CAT, APX and some peroxidases, therefore, a chilling induced increase in POX could be related to defense mechanism of fruit to cope with oxidative stress [20]. Chilling injury due to chilling stress induced ethylene production which in turn might have reduced the level of POX in control fruits and these results were found to be in concordance with those of Ben-Amor *et al* and Maria *et al* [19, 21] who reported ethylene reduced POX activity in chilled cantaloupe melons and Fortune mandarins.

Superoxide dismutase has been detected as an essential defense enzyme against the potential toxicity of ROS. In the unripened fruits, even though CAT and SOD activity increased in all control fruits stored at 8°C for a period of 45 days CI could not be prevented whereas the CI was effectively alleviated in CA storage. These results infer that the levels of SOD and its different forms at the time of harvest continued till the CA storage stabilized to maintain cellular redox homeostasis and could have prevented ROS. The defensive action of SODs against ROS shows age related changes in the present studies agree with senescence of ripening tomato [22]

Phenols also act as antioxidants [23]. Storage environmental factors including temperature stress and CO₂ may affect total phenol content and its types. During CA storage and ripening, total phenol content and DPPH radical scavenging capacity of fruit decreased and may be influenced by storage environment. Decrease in total phenol content was more in air stored control fruits than CA stored fruits. Observations of present study were in agreement with Kem *et al.* [17]. Mechanism for the decrease in total phenol content and DPPH radical scavenging capacity in air-stored control fruits might also be due to CI induced membrane damage resulting from exposure of more phenols to polyphenol oxidases. So CA stored fruits were observed to be better DPPH radical scavenger than air-stored control fruits.

Development of characteristic colour of the mango skin and edible flesh is mostly influenced by the presence of carotenoids. CA stored ripened and air-stored control fruits in the present studies showed increased carotene content. Pre-treatments did not significantly affect the synthesis of carotene during either storage or ripening. Thomas [25] reported that fruits stored at 7°C for 15 days and followed by ripening at room temperature produced 22-53% less carotenoid than those mangoes allowed to ripen normally. Present studies reveal the significant increase in carotene content in CA stored fruits than in air-stored control fruits and this could be due to effective alleviation of CI by CA storage thereby positively influencing carotenoid synthesis.

CONCLUSION

Alphonso mango fruit after CA storage at 8°C and post CA ripening did not show any CI. However, control fruits have shown CI symptoms. All untreated CA stored fruits were rotten but prochloraz and HW pre-treatments prevented the disease incidence. Results presented in this paper showed that pre-treatments and storage environment affect AOE activities, total phenol content and DPPH radical scavenging capacity. CA stored fruits were better retainers of antioxidants and antioxidant enzymes. Therefore, findings of the present study serve as basic information for cellular homeostasis of CA stored and control fruits during storage and ripening.

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Table 1: Effect of CA storage on total phenols, carotene and DPPH radical scavenging activity of fruits during storage at 8°C followed by ripening at ambient conditions.

Storage	After CA storage (45 days at 8 °C)			After ripening (45 days CA stored at 8 °C+ one week at RT)			
	Pre-treatments	Phenols (mg ⁻¹ 100 g ⁻¹ FW)	DPPH (IC50 µg of phenols)	Carotenoids (mg ⁻¹ 100 g ⁻¹ FW)	Phenols (mg ⁻¹ 100 g ⁻¹ FW)	DPPH (IC50 µg of phenols)	Carotenoids (mg ⁻¹ 100 g ⁻¹ FW)
CA storage	Prochloraz	86.6 (68.65) ^b	5.96 ^b	1.60 (7.23) ^c	60.8 (51.30) ^d	9.12 ^d	6.70 (15.00) ^a
	HW-treated	86.2 (68.34) ^b	6.12 ^b	1.72 (7.52) ^c	58.6 (49.96) ^d	9.74 ^d	6.86 (15.18) ^a
Air-stored	Prochloraz	76.6 (61.08) ^c	7.64 ^c	1.82 (7.75) ^c	38.2 (38.17) ^e	12.20 ^e	5.38 (13.36) ^b
	HW-treated	74.4 (59.61) ^c	7.34 ^c	1.84 (7.78) ^c	36.0 (36.86) ^e	12.28 ^e	5.46 (13.48) ^b
	Untreated	75 (60.01) ^c	7.42 ^c	1.82 (7.74) ^c	36.6 (37.22) ^e	12.50 ^e	5.16 (13.11) ^b
At harvest*		100 (85.70) ^a	4.02 ^a	1.02 (5.78) ^c			
Phenol SEM = 1.17, C.V. (%) = 4.70 and CD at 5% = 3.35							
DPPH SEM = 0.29, C.V. (%) = 7.65 and CD at 5% = 0.84							
Carotenoids SEM = 0.32, C.V. (%) = 7.00 and CD at 5% = 0.93							

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