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Synergistic Influence of Pre-Harvest Calcium Sprays and Postharvest Hot Water Treatment on Fruit Firmness, Decay, Bitter Pit Incidence and Postharvest Quality of Royal Delicious Apples (*Malus x domestica* Borkh)

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ABSTRACT

Experiments were conducted to observe the effect of pre-harvest calcium (Ca) applied as calcium chloride (1% W/V) and postharvest hot water treatment (HWT) on "Royal Delicious" apples. For this**,** apples were divided in 4 lots viz., untreated (neither Ca nor HWT), Ca alone (pre-harvest 3 sprays of $CaCl_2$ (1.0% w/v) in the orchard), HWT (42˚C for 2 h). Apples of all four lots were stored in cold storage maintained at 0˚C ± 1˚C and 90% - 95% relative humidity for 6 months. After storage, fruits were removed to ambient conditions for 5 days, and then observations on decay area and incidence, bitter pit incidence, fruit Ca content, lipoxygenase (LOX) and antioxidant (AOX) activity, fruit firmness and fruit quality parameters were recorded. After 6 months in cold storage plus 5 day at 22˚C ± 2˚C and 70% + 4% RH, apples, which received Ca as pre-harvest spray or those which received postharvest hot water treatment or Ca + HWT had significantly lesser decay area (decay lesions) caused by *Penicillium expansum* or *Botrytis cinerea* than untreated ones (control). Ca + HWT treatment was significantly more effective on *B. cinerea* than *P. expansum.* Untreated apples exhibited higher incidence of bitter pit (18.2%) than those treated with Ca or HWT or both. Fruit Ca content (2.92% DM) were significantly lower and conversely the LOX activity (6.9 µmoles $min^{-1} \cdot g^{-1}FW$) was higher in untreated apples. Similarly, total phenolics and AOX activity were also lower in the untreated apples than Ca or HWT treated. HWT or Ca treated apples have beneficial effects on fruit firmness, peel colour and quality parameters like TSS and ascorbic acid content. Thus, it is concluded that pre-harvest sprays of calcium chloride with postharvest HWT is highly useful for "Royal Delicious" for reducing decay loss, maintaining firmness, high levels of antioxidants and fruit quality.

Keywords: Hot Water Treatment; Lipoxygenase Activity; Antioxidant Activity; Bitter Pit; Fruit Quality

1. Introduction

Apple (*Malus x domestica* Borkh) is the most important temperate fruits of the world. Several varieties are grown commercially but "Royal Delicious" dominates others in almost every country [1]. In India, apple accounts for about 75% area among temperate fruits. However, productivity of apple in India is dismally low in comparison to other countries of the world primarily because it is mainly cultivated under rainfed conditions [1]. Apples can be stored for about 6 months in cold stores maintained at 0˚C - 1˚C and 90% - 95% RH and under controlled atmospheric conditions for about 8 months. During prolonged storage, there is softening of fruits, development of several postharvest physiological disorders, and diseases and decline in fruit quality [1]. To reduce such problems, several techniques like the use of fungicides and other protectants have been standardized. Moreover, consumers increasingly demand high-quality commodities be preserved by means other than using techniques or chemicals regarded as unsafe [2-4]. Thus, use of synthetic chemicals to control postharvest diseases in fruits is becoming restricted in several parts of the world [3-6]. Pre-storage heat treatment of apples has been shown to affect quality during storage of "Golden Delicious" apples [5]. Similarly, postharvest heat and calcium treatments have been found quite effective in reducing decay caused by *Botrytis cinerea* in apples, and these effects are enhanced if heated apples are dipped in calcium chloride solutions [4,5,7,8]. For instance, exposing "Spartan" and "Golden Delicious" apple to 38˚C

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for 4 or 6 days and subsequently storing them at 0˚C for 4 - 7 months suppressed softening and naturally occurring decay caused by *Corticium* and *Penicillium* species [8]. Klein *et al*. [9] reported that exposure of "Anna" and "Granny Smith" apples to 38˚C for 4 days, and that a Ca dip after heat treatment augmented the effect of heat appreciably. Similarly, Conway *et al*. [5] reported additive effect of heat treatments (38˚C for 4 days) and infiltration of calcium chloride (3%) on reducing decay and maintaining quality of "Golden Delicious" apples. Holding apples at 38˚C for 4 days or at 42˚C for 1 day after harvest promotes resistance to physiological and pathological disorders [8,10]. Combining the 2 treatments, prestorage heat and Ca dip, gives additional benefit over either alone in maintaining fruit firmness [8] or reducing storage disorders [5]. In all these studies, apples were exposed to hot water at 38˚C for 4 - 6 days or 42˚C for 2 days. Exposing apples to this temperature for 4f6 days appears to be quite expensive and cumbersome. Moreover, in India, the major harvesting season for "Royal Delicious" apples is July-August, depending on the location and elevation of the area when the temperature (35˚C - 38˚C) and RH (70% - 75%) are quite high in comparison to other apple growing countries. Considering the apple growing conditions in India and abroad, we conducted some preliminary studies and found that HWT of "Royal Delicious" apples at 42˚C for 2 or 4 hours is quite effective than exposing them to 38˚C for 2, 4 or 6 days. Furthermore, postharvest $CaCl_2$ treatments to apples are not given in India, however orchardists use 3 - 4 pre-harvest Ca sprays. Hence, considering the usefulness of hot water treatment and Ca in several parts of the world, we undertook this study with the objective to observe the additive effects, if any, of pre-harvest Ca sprays with postharvest hot water dip on softening, decay, occurrence of bitter pit and quality of "Royal Delicious" apples during storage.

2. Materials and Methods

2.1. Experimental Site and Material

The studies were conducted in the Division of Post Harvest Technology, Indian Agricultural Research Institute, New Delhi 110 012 during 2008-10. One lot of "Royal Delicious" apple trees were sprayed with $CaCl_2$ (1.0% w/v) in the orchard at Kullu (Himachal Pradesh) thrice *i.e*. 30, 15, and 7 days before harvest (August 15 every year), and other lot was not sprayed at all. Apples from both lots were harvested at full maturity (starch index 2.5/4.0) and transported to New Delhi. After sorting and grading, fruit of these two lots were subdivided into two sub-lots, and then one lot each was either dipped in hot water (42˚C for 2 h), and other was not. After this, fruit of all four lots (200 each) were stored in cold storage maintained at 0˚C ± 1˚C and 90% - 95% relative humidity for 6 months. After storage, fruit were taken to ambient conditions (22˚C ± 2˚C and 70 ± 4 RH) for 5 days, and then observations on decay rot (%), bitter pit incidence (%), fruit Ca content (% DM) and fruit quality parameters like fruit firmness, TSS, fruit colour, fruit firmness, ascorbic acid content, and acidity etc. were recorded.

2.2. Decay Area and Decay Incidence

Twenty-five fruits from each treatment were wounded on two sides to a depth of 2 mm by pressing them down on the head of a nail 2 mm in diameter [5]. The fruits were then immersed for 15 s in a conidial suspension (10^8 CFU/ml) of *Botrytis cinerea* and *Penicillium expansum*. Both the pathogens were procured from Division of Plant Pathology, Indian Agricultural Research, New Delhi and cultured on media. After extraction, their concentration was made as 10^{-8} CFU/ml and applied to fruits. The area of decay was calculated by means of diameter of the lesions after 5 days at ambient conditions [11].

2.3. Bitter Pit Incidence (%)

The bitter pit incidence was determined by counting the normal and pitted fruits in each lot, replicated three times. The bitter pit incidence was represented as percentage (%).

2.4. Estimation of Fruit Ca Contents

Ca contents in the fruit were determined by adopting the procedure of Sharma and Singh [12]. First 2-mm layer of the peel and outer flesh of each fruit was removed with a mechanical peeler and discarded, and then the core was removed. The remaining fruit part was used for the estimation of Ca content. After ashing, the residue was dissolved in nitric acid to a final solution concentration of 0.16 M. Fruit calcium content was measured by atomic absorption spectrophotometer (AAS 4141; ECO Ltd., New Delhi, India). From each fruit, 3 - 4 mm thick longitudinal slices were taken, reducing the total weight of the sample to 50 - 60 g.

2.5. Estimation of Lipoxygenase Enzyme (EC1.13.11.12) Activity

2.5.1. Preparation of Substrate

The substrate was prepared as per the procedure described for strawberry by Sharma *et al.* [13] with slight modifications. First, linolenic acid (0.1 ml) was dissolved in 1 ml 0.1 N NaOH solution. To this, 150 µL Triton-X-100 was added. The solution was emulsified in an Ultra

Turrax for 2 min, and diluted to 50 ml with distilled water. Similarly, blank was prepared by using substrate solution. The substrate and the blank were stored at 4˚C in dark until use.

2.5.2. Preparation of Crude Enzyme Extract

Crude enzyme extract was prepared at 4˚C, following the method of Sharma *et al*. [13] with minor modifications. Diced 1 g apple fruit was homogenized in pre-chilled pestle and mortar by mixing in ice cooled 10 ml EDTA. The homogenate was centrifuged at 15,000 × g for 20 min at 4˚C and supernatant was used for assay of lipoxygenase activity.

2.5.3. Measurement of Lipoxygenase Activity

Enzyme assay was carried out as per the procedure of Sharma *et al*. [13] with minor modifications. First, 50 µL of enzyme extract was added to 2.50 ml of substrate solution in a cuvette, mixed thoroughly and absorbance was recorded at 234 nm in spectrophotometer (Perkin-Elmer UV-VIS Lambda-25; San Jose, California, USA) for 3 min at 30 sec interval. LOX activity was expressed as "µmoles $min^{-1} \cdot g^{-1}$ FW".

2.6. Total Phenols and Antioxidant Activity

The total phenolic content of the apples were determined by the method of Singleton and Ross [14] method with some modifications. 5 g fruit was crushed in 10 ml of 80% ethanol and centrifuged. The homogenate was centrifuged at 15,000 × g for 20 min at 24˚C and supernatant was used for assay of total phenols. 0.5 ml of the sample was added to 2.5 ml of 0.2 N Folin-Ciocalteau reagent and placed for 5 min. 2 ml of 20% Na_2CO_3 was then added and the total volume made up to 25 ml using 80% ethanol. The above solution was then kept for incubation in boiling water bath for 15 min till it becomes blue-black. Absorbance was measured at 760 nm using 1 cm cuvette in spectrophotometer (Perkin-Elmer UV-VIS Lambda 25). Gallic acid (0 - 800 mg/L) was used to produce standard calibration curve. The total phenol content was expressed in mg of gallic acid equivalents (GAE)/100 g of extract. Antioxidant capacity in apples was determined by following CUPRAC method, which was standardized by Apak *et al.* [15] and represented as µmoles Trolox g^{-1}.

2.7. Determination of Fruit Quality Parameters

2.7.1. Fruit Firmness

Fruit firmness was determined using a texture analyzer (model: TA + Di, Stable micro systems, UK) using compression test. The fruit was punched at the tip, middle and stem-end point with 2 mm diameter probe up 20 mm depth [13]. Fruit firmness was expressed as N (Newton).

2.7.2. Fruit Peel Colour

Changes in peel colour were determined by using chromometer where the CIE lab system (L^*, a^* and b^*) was used [16]. Interpretation of the L^*, a^* and b^* values followed those of where L^* = the lightness of the colour with zero for black and 100 for white, a^* = red (positive) or green (negative) and b^*= yellow (positive) or blue-yellow (negative).

2.7.3. Physico-Chemical Attributes

TSS (%) was recorded with hand refractometer. Acidity was determined by titrating known amount of fruit juice with 0.1N NaOH solution. Ascorbic acid contents (mg/100 g pulp) were also determined by following standard procedure [17]. For recording all these parameters, 5 fruits were randomly selected from a single lot, which was replicated 5 times.

2.8. Statistical Design and Analysis of Data

Two years data were pooled and results were subjected to analysis using Duncan's multiple range test for completely randomized block design and the treatment means were compared using the least significant difference (LSD) values at a significance level of $P \leq 0.05$. All analyses were conducted following the procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC, USA [18].

3. Results

3.1. Effect on Decay Area and Incidence

After 6 months in cold storage plus 5 day at 22˚C ± 2˚C and 70% + 4% RH, apples, which received Ca as preharvest spray or those which received postharvest hot water treatment (42˚C for 2 h) or their combination had significantly lesser decay area (decay lesions) caused by *Penicillium expansum* or *Botrytis cinerea* than those which were not treated at all (control). Such effects of different treatments were much more pronounced on *B. cinerea* than *P. expansum* (**Table 1**). Hot water treated apples had lesser decay area than Ca treated apples. However, additive effect of Ca + hot water was most effective in reducing the decay area caused by *P. expansum* (226 mm^2) and *B. cinerea* (62 mm^2) over their individual effects. Further, the decay incidence was cent percent in untreated apples, which was significantly reduced by Ca or HWT but the best results were obtained by combined effects of Ca and hot water treatment, which reduced the decay caused by *P. expansum* (22.2%) and *B. cinerea* (10.6%) over control (100%) (**Table 1**).

Table 1. Effect of pre-harvest Ca sprays and postharvest hot water treatments on decay incidence and decay area in "Royal Delicious" apples.

Treatment	Decay area (mm^2)		Decay incidence (%)		Bitter bit incidence (%)
	Penicillium expansum	*Botrytis cinerea*	*Penicillium expansum*	*Botrytis cinerea*	
Neither Ca spray nor HWT	880[a]	520[a]	100[a]	100[a]	18.2[a]
Pre-harvest Ca spray only	410[b]	280[b]	88.6[b]	78.2[b]	4.3[c]
Post-harvest HWT only	380[c]	166[c]	43.6[c]	18.4[c]	7.4[b]
Pre-harvest Ca + postharvest HWT	226[d]	62[d]	22.2[d]	10.6[d]	1.0[d]
$LSD_{0.05}$	12.5	9.6	7.2	6.1	2.3

[*]Means within columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test. Data are mean + SE of 5 fruit per treatment with 3 replications.

3.2. Bitter Pit Incidence

Untreated apples exhibited higher incidence of bitter pit (18.2%) than treated ones, although hot water treated apples had higher incidence of bitter pit (7.4%) than those which received Ca (4.3%). However, additive effect of Ca and hot water treatment was observed on apples which reduced bitter pit incidence to 1.0% (**Table 1**).

3.3. Fruit Ca Content and LOX Activity

Calcium content in apples was least in untreated apples (2.92%) than treated apples. Although fruit which received Ca as pre-harvest sprays contained higher concentration of Ca (3.76%) than those which received hot water treatment (3.10%). However, synergistic effect of Ca and hot water treatment was evident as such apples had maintained significantly higher Ca level (4.12%) during storage (**Table 2**). Conversely, LOX activity was higher in untreated apples (6.9 μmoles $min^{-1}\cdot g^{-1}FW$) than treated ones whether with Ca (5.4 μmoles $min^{-1}\cdot g^{-1}FW$) or HWT (4.8 μmoles $min^{-1}\cdot g^{-1}FW$) alone or in combination (3.2 μmoles $min^{-1}\cdot g^{-1}FW$). Combined treatment was much effective in reducing the LOX activity over untreated ones (**Table 2**).

3.4. Total Phenols and Antioxidant Capacity

Untreated apples have significantly lower level of phenolic compounds (35.2 mg GAE/100 g fruit) and antioxidant capacity (26.2 μmoles Trolox g^{-1}) than those receiving Ca or HWT alone. Apples, which received both Ca and HWT had very high level of phenolic compounds (49.6 mg GAE/100 g fruit) and antioxidant capacity (46.2 μmoles Trolox g^{-1}) (**Table 2**).

3.5. Fruit Quality Parameters

3.5.1. Fruit Firmness

The synergistic effects of combined hot water and Ca treatment in maintaining firmness of apples during storage was evident as the firmest apples (31.8 N) were those which received both Ca and hot water treatment (**Table 3**). Apples which received either Ca or heat, although were firmer than untreated apples (control) (18.3 N) but were less firm than those treated with Ca and hot water before storage (**Table 3**).

3.5.2. Peel Colour

Untreated apples had lower "a" (37.4) and higher "b" (39.3) values than treated apples indicating that untreated apples had developed less red colour on their peel, and had more yellow colour than those which received either Ca or hot water treatment indicating that such apples were far superior in maintaining attractive red colour than untreated apples during and after the storage (**Table 3**).

3.5.3. Physico-Chemical Attributes

Untreated apples were far below inferior in all the quality parameters like TSS or ascorbic acid contents than those treated with Ca or hot water alone or in combination. Apples which received both Ca and hot water treatment retained significantly higher TSS (16.4%), ascorbic acid content (30.6 mg/100 g pulp) and acidity (1.42%) than untreated apples or those which received Ca or hot water treatment alone, and thus were far superior in all quality attributes (**Table 3**). TA remained unaffected either treatment.

4. Discussions

Our results showed that pre-harvest Ca and postharvest hot water treatment reduced both decay area and decay incidence significantly over untreated apples, and combined treatment of Ca and HWT was more effective than their individual effects, which is similar to the decay reduction observed with *Penicillium expansum* by Sam *et al*. [19] and with *Botrytis cinerea* by Klein *et al*. [4] and

Table 2. Effect of pre-harvest Ca sprays and postharvest hot water treatment on fruit Ca contents, LOX activity, phenolics and antioxidant activity in "Royal Delicious" apples.

Treatment	Fruit Ca content (%)	LOX activity (μmoles $min^{-1} \cdot g^{-1}$ FW)	Total phenolic content (mg/100 g fruit)	Antioxidant activity (μmoles Trolox g^{-1})
Neither Ca spray nor HWT	2.92[d]	6.9[a]	35.2[d]	26.2[c]
Pre-harvest Ca spray only	3.76[b]	5.4[b]	39.5[c]	35.8[b]
Post-harvest HWT only	3.10[c]	4.3[c]	41.2[b]	36.4[b]
Pre-harvest Ca + postharvest HWT	4.12[a]	3.2[d]	49.6[a]	46.2[a]
C.D. ($P \leq 0.05$)	0.72	0.63	1.2	2.6

*Means within columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test. Data are mean + SE of 5 fruit per treatment with 3 replications.

Table 3. Effect of pre-harvest Ca sprays and postharvest hot water treatments on fruit firmness, peel colour and some quality parameters of "Royal Delicious" apples.

Treatments	Parameter						
	Fruit firmness (N)	Peel colour			TSS (%)	Ascorbic acid content (mg/100 g pulp)	Titratable acidity (%)
		L*	a*	b*			
Neither Ca spray nor HWT	18.3[c]	42.2[a]	37.4[d]	39.3[a]	13.2[b]	18.2[d]	1.37[b]
Pre-harvest Ca spray only	24.1[b]	39.4[b]	41.6[c]	32.4[b]	15.6[b]	22.2[c]	1.39[b]
Post-harvest HWT only	26.1[b]	38.4[b]	48.3[b]	30.5[b]	15.9[b]	29.6[b]	1.41[a]
Pre-harvest Ca + postharvest HWT	31.8[a]	32.3[b]	52.5[a]	25.8[c]	16.4[a]	30.6[a]	1.42[a]
$LSD_{0.05}$	2.23	2.56	3.32	2.66	0.56	1.82	NS

*Means within columns followed by the same letter do not differ significantly at $P = 0.05$ according to Duncan's multiple range test. Data are mean + SE of 5 fruit per treatment with 3 replications.

Conway *et al*. [5]. Exposure of fruit to temperature > 38˚C for 4 d have been reported to prevent scald [11] but did not significantly reduced decay. In previous studies, HWT temperature was reported to be 38˚C and exposure time as 4 days. However, our study differs from the previous ones in exposure time (2 h) and HWT temperature (42˚C). Ca alone did influence the decay area and incidence considerably indicating that Ca in the form of calcium chloride has fungistatic effect on reducing the decay in Royal Delicious apples caused *B. cinerea* and *P. expansum*. The mechanism by which Ca reduces decay may be related to Ca ions in cell wall [20,21]. Cell wall pectins are primarily composed of four-linked galactoronosyl residues, and the stability of cell wall may be related to its co-operative binding with Ca ions [22], making the cell wall of apples less accessible to enzymes that cause softening or to cell wall degrading enzymes produced by pathogens. Increasing the concentration of Ca in apple cell wall has been shown to inhibit polyglacturonase activity produced by *P. expansum* [23,24]. Although, the optimum concentration of Ca in fruit tissue may vary and accordingly the decay incidence caused by different pathogens.

The mechanism by which hot water treatment reduces decay can't be explained satisfactorily. However, some authors believe that inactivation of pectin degrading enzymes by HWT is a possible mechanism [4,5] as heat treated "Golden Delicious" and "Spartan" apples have shown lower levels of soluble pectin than untreated ones [8,25]. Further, HWT may inhibit protein synthesis required for cell wall degradation and ethylene synthesis [26]. Prestorage heat had higher effect on fruit firmness than Ca treatment, primarily because Ca may not have reached in enough concentration in the cortex region to influence firmness [9] or its concentration might have reduced considerably during storage.

Higher incidence of bitter pit in untreated apples than Ca or hot water treated apples could be attributed to lower retention of Ca in untreated apples than those which received Ca or HWT. Similarly, untreated apples have higher LOX activity than HWT or Ca treated apples, indicating that untreated apples were more senescent than treated ones. This can be linked with the Ca content and LOX activity of the treated and untreated apples. It is well known that calcium plays vital role in preventing various physiological disorders, and LOX activity has

strong relationship with fruit senescence and physiological disorders [12,27,28]. Although, the mechanism by which calcium prevents physiological disorders is not well understood, however it is known that it principally acts on middle lamella of cell wall and play its role of cross-linking [29,30], where it may influence membrane bound enzymes, like lipoxygenase (LOX), that converts the unsaturated fatty acids like linoleic and linolenic to their hydroperoxidate derivatives [30,31]. Although, LOX is found in tissues of a wide variety of higher plants, there are many questions with regard to its functions in plant lipid metabolism, yet scientists world over largely agree that it is responsible for the typical breakdown of linolenic acid, and thus responsible for the development of some physiological disorders [32], which demonstrates the participatory role of LOX in fruit senescence. Postharvest heat treatment has been reported to reduce storage disorders such as superficial scald and bitter pit [7,33]. Combining the two treatments, pre-storage heat and calcium dip, gives additional benefit over either alone in decreasing storage disorders [25,26], which supports our findings as well.

Untreated apples have significantly lower level of phenolic compounds, AOX capacity, lower colour values and were inferior in quality attributes like TSS, ascorbic acid content than those receiving Ca or HWT alone or both. Similarly, Ca or HWT treated apples were firmer than untreated ones. There is no explanation as to how Ca or HWT treated apples have higher ascorbic acid content than untreated ones but higher AOX capacity can be attributed to higher ascorbic acid content and phenolic compounds. Moreover, retention of high Ca content both in Ca or HWT treated apples might have contributed to high AOX activity. Further, better firmness of Ca or HWT treated apples can be explained by the fact that pectin degrading enzymes are inactivated as a result of such treatments [Conway *et al*., 5 and Poritt and Lidster, 9]. Conway *et al*. [5] and Klein and Lurie [25] have also reported better firmness and quality of heat or Ca treated apples.

5. Conclusion

Our results revealed that pre-harvest Ca sprays and postharvest HWT treatment (42˚C for 2 h) helps in reducing the spoilage and bitter pit incidence while maintaining the Ca level, antioxidant capacity, fruit firmness and quality of "Royal Delicious" apples during storage.

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