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ORIGINAL ARTICLE

Post-harvest calcium chloride treatments influence fruit firmness, cell wall components and cell wall hydrolyzing enzymes of Ber (*Ziziphus mauritiana* Lamk.) fruits during storage

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Abstract Ber fruits of two varieties having variable shelf lives viz. Umran (8–9 days) and Kaithali (4–5 days) given post-harvest treatments of calcium chloride (1% and 2%) were analyzed for various cell wall components, cell wall hydrolyzing enzymes and fruit firmness at 2 days interval until complete decay. There was a continuous decrease in cellulose, hemicellulose and pectin contents during storage in both the varieties with more reduction in Kaithali, a variety having short shelf-life. The decline in cell wall components was accompanied by parallel increase in activities of cellulase, polygalacturonase (PG) and pectin methylesterase (PME). Post-harvest treatment of Ber fruits with calcium chloride resulted in significantly lowering of activities of cellulase (20–22%), PG (23–29%) and PME (25–28%) thereby retaining higher cell wall components viz. cellulose (9–11%), hemicellulose (7–8%) and pectin (12–13%) as compared to their respective control in both the varieties. The delay in cell wall hydrolysis, as mediated by calcium chloride corresponded to the higher retention of fruit firmness.

Keywords Ber · Cellulase · Cellulose · Hemicellulose · Pectin methylesterase · Polygalacturonase

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Introduction

Ber (*Ziziphus mauritiana* Lamk.) is one of the important tropical fruits of arid and semi-arid India. It is a nutritious fruit that contains vitamin A, B complex, C; minerals like calcium, iron, phosphorus, potassium, carotenoids and sugars. Being a climacteric fruit, Ber is very perishable and susceptible to skin browning. After harvest, a number of physiological and biochemical changes including synthesis and degradation of pigments, conversion of starch to sugars, hydrolysis of cell wall constituents continue and result in the reduction of quality parameters such as firmness, chlorophyll content, nutrients, incidence of decay and off-flavor. The inherent short shelf-life and spoilage during storage and transportation lead to huge post-harvest losses. Therefore, development of practical solutions to post-harvest problems requires the understanding of biochemical mechanism of ripening-related changes which continue post- ripening.

Fruit softening by enzymatic hydrolysis of cell wall components like cellulose, hemicellulose, pectins and glycoproteins (Ali et al. 2004; Romanazzi et al. 2016) is characterized by the disassembly of the primary cell wall (Giongo et al. 2013), alteration in cellulose microfibrils (Carpita and Gibeaut 1993), modification of hemicellulose (Brummell and Harpster 2001) and the depolymerization of pectin (Rose et al. 1998). The coordinated action of cell wall hydrolyzing enzymes results in loss of integrity of the cell wall (Deng et al. 2005) by the disassembly of the cellulose-hemicellulose network (Cheng et al. 2009).

Calcium ions perform multiple roles in plant physiology. Treatment of fruits with calcium may postpone senescence by maintaining membrane integrity and increasing firmness of cell wall (Ishaq et al. 2009; Zhi et al. 2017). Though the research on the effect of calcium in

improving the storage life of fruits and vegetables has been started long back but it was limited to physico-chemical and quality parameters of fruits and vegetables. Moreover, information on the effects of calcium on cell wall components and mechanisms involved in the disintegration during storage is very scanty (Langer et al. 2019; Zhi et al. 2017). We report here the alterations in the cell wall components and cell wall hydrolyzing enzymes and their correlation with the firmness and shelf-life in two varieties of Ber differing in their keeping quality.

Materials and methods

Fruit source, treatments and storage conditions

Two varieties of the Ber fruits, Kaithali (shelf-life 4–5 days) and Umran (shelf-life 8–9 days) were harvested at mature-green stage from the Horticulture Farm, CCS Haryana Agricultural University, Hisar. The fruits of uniform size and color were selected and divided into three groups. Fruits of one group were dipped in aqueous solution of 1% calcium chloride and the second in 2% calcium chloride for 5 min each. The fruits of third group (control) were dipped in water for the same duration. The fruits were then air-dried and stored in cardboard boxes at room temperature. They were then analyzed at two day interval until complete decay. All the chemicals and reagents used in the study were of analytical grade and procured from E. Merck (Bombay), Himedia Laboratories Limited (Bombay), Sigma Chemical Company, (USA), and Sisco Research Laboratories Pvt. Ltd., (Bombay).

Firmness

Flesh firmness was measured by hand held fruit pressure tester penetrometer, using cylindrical plunger of 8 mm diameter and firmness scale of 13 kg/cm^2. The firmness was recorded from each side of the equatorial region of the fruit. Firmness of five fruits per treatment was measured and it was expressed in kg/cm^2.

Cell wall components

Cellulose and hemicellulose

Cellulose and hemicellulose were estimated by the method of Van Soest and Wine (1967) modified by Pradhan and Bhatia (1986). To estimate cellulose, acid detergent fibres (ADF) was first estimated by refluxing 1.0 g sample with 100 mL ADF reagent containing 0.5 g cetrimide and 2.8 mL concentrated sulphuric acid for 1 h. After the removal of ADF, the residue was taken in a sintered glass crucible and washed twice by stirring with 72% H_2SO_4 (w/v). The crucible filled with acid was kept for 3 h and then acid was removed. The residual contents were washed with hot water in order to make them acid-free. They were then dried for 24 h at 100 °C and re-weighed. The loss in weight was expressed as cellulose content.

To quantify hemicellulose, neutral detergent fibres (NDF) were first determined by refluxing 1.0 g sample with 100 mL NDF reagent containing 1.0 g sodium lauryl sulphate, 1.861 g EDTA, 0.68 g borax (sodium borate decahydrate), 0.46 g disodium hydrogen phosphate and 1.0 mL ethylene glycol monoethyl ether for 1 h. The solution was filtered through sintered glass crucible. The left-over residues were washed with hot distilled water and then with acetone, dried for 24 h at 100 °C and re-weighed. The residue represented NDF content. Hemicellulose content in the sample was calculated by the difference between NDF and ADF.

Pectin

To extract pectin, 1.0 g Ber fruit was agitated with 25 mL H_2SO_4 (72%, w/v) for 30 min and volume was made to 100 mL with distilled water (Ahmed and Labavitch 1978). Pectin content was quantified by estimating uronic acid content as reported by Blumenkrantz and Asboe-Hansen (1973). The extract (0.2 mL) was mixed with borax solution (2 mL) and kept for 5 min. After shaking, the mixture was incubated in boiling water bath for 10 min and cooled. To the mixture, 20 µL metahydroxydiphenyl solution (1.5 g/L in 12.5 mM NaOH) was added and content were shaken until pink color appeared. The volume was made to 5 mL with conc. H_2SO_4 and absorbance was read at 520 nm. The amount of uronic acid was calculated from a standard curve prepared by D-galacturonic acid (50–250 µg/mL).

Cell wall-degrading enzymes

Pectin methylesterase (EC 3.1.1.11)

Pectin methylesterase was extracted by the method of Hagerman and Austin (1986). One gram of fruit tissue was macerated in 5 mL Tris–HCl buffer (0.1 M, pH 7.5) containing 10% NaCl, centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was used as an enzyme extract. The reaction mixture contained 2.5 mL apple pectin (0.5%), 0.4 mL bromothymol blue (0.01%) and enzyme extract (0.1 mL). The change in the absorbance at 620 nm for 30 min was converted to galacturonic acid from the standard curve (50–500 µg/mL) prepared under the same assay conditions. One enzyme unit was defined as mg of galacturonic acid in 30 min.

Polygalacturonase (EC 3.2.1.15)

Extraction of polygalacturonase was performed at 4 °C as described by Singh and Singh (1993). One gram of fruit sample was homogenized in 0.1 M sodium acetate buffer (pH 5.2) containing 0.02 M sodium metabisulphite and 10% NaCl. The extract was centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was dialyzed against 0.01 M sodium acetate buffer (pH 5.2) for 4 h by changing the buffer every hour. The enzyme was assayed following the method of Ahmed and Labavitch (1978). The assay mixture (1 mL) contained 0.2 mL sodium acetate buffer (0.1 M, pH 5.2), 0.5 mL polygalacturonic acid (3 g/L) and 50 µL of 125 µg each of chloramphenicol and cycloheximide and 0.2 mL enzyme extract. The mixture was incubated for 20 h at 37 °C. Reaction was terminated in a boiling water bath by heating the tubes for 10 min. Reducing sugars released were estimated by method of Nelson (1944) as modified by Somogyi (1952) using galacturonic acid (100–500 µg/mL) as standard. One unit of enzyme was defined as 1.0 mg of galacturonic acid released in 20 h.

Cellulase (EC 3.2.1.4)

Cellulase extraction and assay system were the same as for polygalacturonase except that 0.5% carboxymethyl cellulose sodium salt was used as substrate instead of polygalacturonic acid. The reaction was started by addition of 0.5 mL substrate solution and terminated in a boiling water bath by heating the tubes for 10 min. Reducing sugars were estimated by method of Nelson (1944) as modified by Somogyi (1952) using glucose (40–200 µg/mL) as standard. One unit of enzyme was defined as mg of glucose released in 20 h.

Statistical analysis

All experiments were replicated three times and data were analyzed using OPSTAT software (Sheoran et al. 1998), CCS Haryana Agricultural University.

Results and discussion

Firmness

Firmness of the fruit is one of the indices for determining the maturity of the fruits. It is also important in assessing the resistance of fruits to mechanical damage. Firmness is mainly due to pectin polymers (Fertonani 2006), cross linked to ions, mainly Ca^{2+}, which maintains adjacent chains bonded among themselves (White and Broadley 2003). Firmness of Ber fruit decreased progressively with the increasing storage period from 7.13 kg/cm^2 at 0 DOS (days of storage) to 4.2 kg/cm^2 at 12 DOS stage and from 6.57 kg/cm^2 at 0 DOS stage to 4.67 kg/cm^2 at 8 DOS stage in control fruits of Umran and Kaithali, respectively (Fig. 1). The fruits treated with $CaCl_2$ had significantly higher firmness as compared to the control fruits of both varieties. However, there was no significant effect of $CaCl_2$ concentration on fruit firmness. These results are in accordance with the results of Benavides et al. (2002) and Casero et al. (2004) in apple, Ishaq et al. (2009) in apricot and Zhi et al. (2017) in peach and suggested that post-harvest application of calcium decreased softening and maintained fruit firmness during storage. The retention of firmness in calcium treated fruits might be due to its accumulation in the cell walls leading to facilitation in the cross linking of the pectic polymers which increases wall strength and cell cohesion (White and Broadley 2003).

Cell wall components

Generally, the formation of the cellulose–hemicellulose network with hydrogen-bond crosslinks between hemicelluloses and cellulose microfibrils provides strength to the cell wall (Cheng et al. 2009). Pectin is one of the main components of the middle lamella and primary cell walls, and a major adhesive material for pectic polysaccharides and other cell wall components in plant tissues (Duan et al. 2008).

The cellulose content decreased linearly in both the cultivars during the storage (Fig. 2a). In Umran, it declined from 19.37% at 0 DOS stage to 9.73% at 8 DOS and further to 7.11% at 12 DOS and in Kaithali, it decreased from 15.01% at 0 DOS stage to 7.25% at 8 DOS stage. However, pretreatment of fruits with $CaCl_2$ resulted in better retention of cellulose at all the stages of storage in both the varieties which is evident from higher mean value of cellulose content in $CaCl_2$ treated fruits of both Umran (about 15.6%) and Kaithali (about 12.1%) as compared to their respective controls (14.3% and 11.1%) at 8 DOS stage.

Hemicellulose content (Fig. 2b) showed continuous decrease throughout the storage period from 9.69 to 1.82% at 12 DOS in Umran and 11.37–4.4% at 8 DOS in Kaithali in control fruits. However, the decrease was much lower in $CaCl_2$ treated fruits (9.69–2.40% in Umran and 11.37–5.15% in Kaithali). The pretreatment of fruits with $CaCl_2$ significantly lowered the rate of decrease of hemicellulose from 3.57 to 3.20% and 7.66–7.15% at 8 DOS in Umran and Kaithali fruits, respectively.

Critical perusal of the data revealed that pectin content declined during storage in both the varieties (Fig. 2c). However, the pretreatment of fruits with $CaCl_2$ resulted in delay in pectin loss thereby maintaining higher pectin

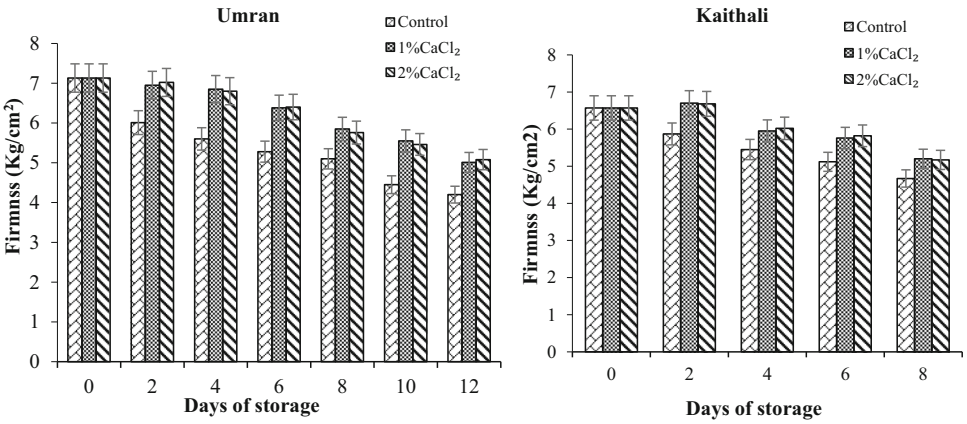

Fig. 1 Effects of $CaCl_2$ on firmness during storage in Ber fruit. The bar (*I*) denotes ± SE. In Umran [CD ($P \leq 0.05$) 0.301 (days of storage), 0.197 (treatments), NS (interactions)]; In Kaithali [CD ($P \leq 0.05$) 0.302 (days of storage), 0.234 (treatments), NS (interactions)]

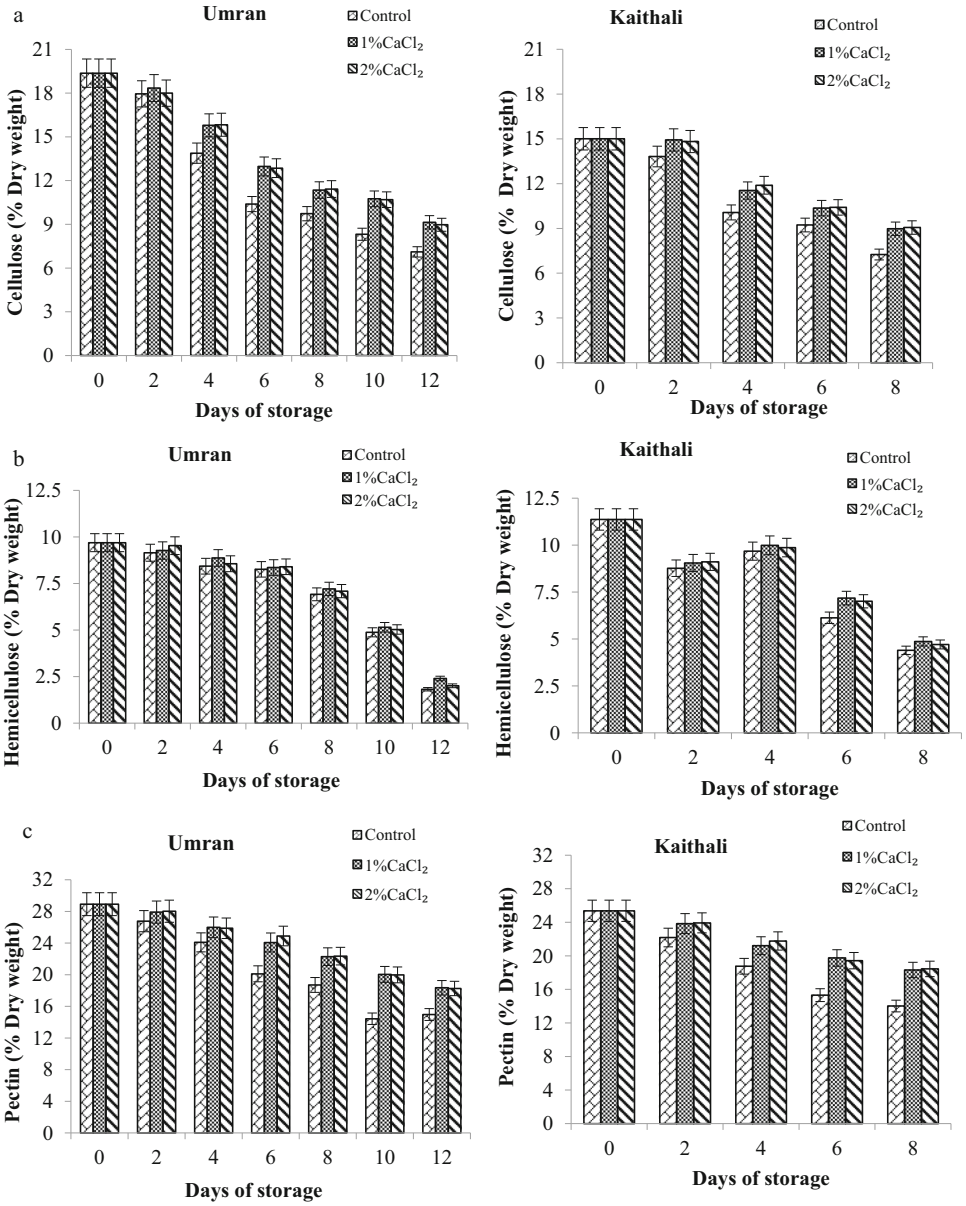

Fig. 2 **a** Effects of $CaCl_2$ on cellulose content during storage in Ber fruits. The bar (*I*) denotes ± SE. In Umran [CD ($P \leq 0.05$) 1.391 (days of storage), NS (treatments), NS (interactions)]; In Kaithali [CD ($P \leq 0.05$) 1.367 (days of storage), NS (treatments), NS (interactions)]. **b** Effects of $CaCl_2$ on hemicellulose content during storage in Ber fruits. The bar (*I*) denotes ± SE. In Umran [CD ($P \leq 0.05$) 1.5261 (days of storage), 0.999 (treatments), NS (interactions)]; In Kaithali [CD ($P \leq 0.05$) 1.547 (days of storage), NS (treatments), NS (interactions)]. **c** Effects of $CaCl_2$ on pectin content during storage in Ber fruits. The bar (*I*) denotes ± SE. In Umran [CD ($P \leq 0.05$) 1.484 (days of storage), 0.972 (treatments), NS (interactions)]; In Kaithali [CD ($P \leq 0.05$) 0.946 (days of storage), 0.732 (treatments), NS (interactions)]

content at all the stages of storage. Fruits treated with 1% $CaCl_2$ retained 22.58% more pectin in Umran and 31.60% in Kaithali fruits at 12 DOS stage and 8 DOS stage respectively. Softening-related changes in pectin, hemicelluloses and cellulose during post-harvest ripening have also been reported in papaya (Manrique and Lajolo 2004), banana (Duan et al. 2008) and grape (Deng et al. 2005). Concomitant with our results, cell wall components were found to be significantly maintained by pretreatment of $CaCl_2$ in strawberry (Lara et al. 2004; Langer et al. 2019), blueberries (Angeletti et al. 2010) and apple (Chardonnet et al. 2002) fruits during storage. Post-harvest calcium treatment seems to increase the cell wall pectin characteristics and to maintain integrity of cell wall by reducing the solubilization of uronic acids in the pectin (Zhi et al. 2017) and disassembly of cellulose-hemicellulose network (Tsantili et al. 2002).

Cell wall hydrolyzing enzymes

Contrary to cell wall components, the activities of cell wall hydrolyzing enzymes increased during storage. As shown in Table 1, the pectin methylesterase (PME) activity increased continuously from 6.46 to 68.14 unit g^{-1} fresh weight in Umran at 12 DOS stage and from 8.35 to 53.81 unit g^{-1} fresh weight in Kaithali at 8 DOS stage. The basal level of PME was much higher (29.3%) in Kaithali than in Umran. Fruits treated with both the concentrations of $CaCl_2$ showed lower PME activity in comparison to their controls and the reduction caused by 1 and 2% of $CaCl_2$ was almost similar as is evident from respectively 25.1–25.8% and 28.2–29.6% decrease in activity in Umran and Kaithali. Similar to our results, delay in loss of PME activity in calcium treated peach fruits could help to retain higher pectin content thus inhibiting loss of cell wall integrity (Zhi et al. 2017). Contrarily, the calcium treatment though enhanced PME activity but maintained higher pectin amount in strawberries during storage (Langer et al. 2019). Reduction in the PME by $CaCl_2$ could be due to the formation of salt-bridge cross links between Ca^{2+} ions and COO^- groups from the pectin content of the fruits (Luna-Guzman and Barrett 2000; Saftner et al. 2003) which makes the cell wall less accessible to the softening enzymes.

Table 1 Effects of $CaCl_2$ (%) treatments on pectin methylesterase (unit g^{-1} FW) activity during storage in Ber fruits

DOS	Umran[a]				Kaithali[b]			
	Nil	1	2	Mean	Nil	1	2	Mean
0	6.46	6.46	6.46	6.46	8.35	8.35	8.35	8.35
2	10.52	8.73	9.13	9.46	11.92	9.35	9.41	10.23
4	21.73	15.82	16.11	17.89	26.32	16.49	16.01	19.61
6	34.21	25.17	26.34	28.57	42.18	29.03	30.21	33.81
8	46.39	32.41	31.39	36.73	53.81	37.21	38.49	43.17
10	51.92	38.78	39.91	43.54				
12	68.14	50.32	49.78	56.08				
Mean	34.20	25.38	25.59		28.52	20.09	20.49	

CD ($P \leq 0.05$)

DOS days of storage

[a]Days of storage 1.425, treatments 0.933, interactions 2.469

[b]Days of storage 1.428, treatments 1.106, interactions 2.473

Like PME, Polygalacturonase (PG) also enhanced during storage in both the varieties and highest PG activity was recorded in the control samples of Umran (66.21 unit g^{-1} fresh weight) and Kaithali (70.76 unit g^{-1} fresh weight) at 12 DOS and 8 DOS stage, respectively (Table 2). Pretreatment with 1% $CaCl_2$ resulted in lowering of PG activity in both the cultivars (50.45 unit g^{-1} fresh weight in Umran and 50.30 unit g^{-1} fresh weight in Kaithali). This may be due to $CaCl_2$ mediated reduction in the enzymes level and increase the neutral sugars in fruits (Langer et al. 2019; Manganaris et al. 2005). Exogenously applied calcium binds the negative charges of deesterified uronic acid residues generated by PME during ripening and therefore enhancing the tissue's mechanical strength (Marzouk and Kassem 2011). Previous research done on freshly cut cantaloupes has shown that $CaCl_2$ treatment improves the fruit firmness and quality (Luna-Guzman and Barrett 2000).

Cellulase activity increased from 18.60 unit g^{-1} fresh weight at 0 DOS to 60.29 unit g^{-1} fresh weight at 10 DOS and declined thereafter to 47.51 unit g^{-1} fresh weight at 12 DOS stage in Umran but it increased continuously from 18.67 unit g^{-1} fresh weight at 0 DOS to 65.59 unit g^{-1} fresh weight at 8 DOS stage in Kaithali (Table 3). Cellulase inhibition at later stage (12 DOS) in Umran may be due to the influx of internal calcium and thus inhibiting the enzyme (Jawandha et al. 2009). Pretreatment of fruits resulted in decrease in cellulase activities at all the stages of storage in both the cultivars. Pretreatment with 1% $CaCl_2$ had more pronounced effect on cellulase activity in both the cultivars as compared to that of 2% $CaCl_2$. The reduction in cellulase, PG activity and other cell wall modifying enzymes by calcium treatment could delay disassembly of cell wall polysaccharides thus maintaining (Zhi et al. 2017) its firmness.

It is evident from our results that Kaithali had higher activity of each of the cell wall hydrolyzing enzymes at all the stages of storage as compared to the firm variety Umran. The faster hydrolysis of the cell wall in Kaithali could be responsible for its short shelf-life. The decline in the activity of cell wall degrading enzymes following

Table 2 Effects of $CaCl_2$ (%) treatments on polygalacturonase (unit g^{-1} FW) activity during storage in Ber fruits

DOS	Umran[a]				Kaithali[b]			
	Nil	1	2	Mean	Nil	1	2	Mean
0	32.15	32.15	32.15	32.15	38.91	38.91	38.91	38.91
2	42.08	38.14	37.22	39.15	46.92	40.29	41.73	42.98
4	52.24	37.68	38.82	42.91	58.87	41.29	42.20	47.45
6	56.40	45.57	46.60	49.52	80.29	55.01	59.96	65.09
8	84.30	63.44	67.06	71.60	128.82	75.98	78.38	94.39
10	97.50	67.61	69.35	78.15				
12	98.78	68.56	69.12	78.82				
Mean	66.21	50.45	51.47		70.76	50.30	52.24	

CD ($P \leq 0.05$)

DOS days of storage

[a]Days of storage 1.586, treatments 1.038, interactions 2.748

[b]Days of storage 1.592, treatments 1.233, interactions 2.758

Table 3 Effects of $CaCl_2$ (%) treatments on cellulase (unit g^{-1} FW) activity during storage in Ber fruits

DOS	Umran[a]				Kaithali[b]			
	Nil	1	2	Mean	Nil	1	2	Mean
0	18.60	18.60	18.60	18.60	18.67	18.67	18.67	18.67
2	19.63	18.02	16.25	17.97	19.56	17.25	17.20	18.00
4	31.87	22.96	21.55	25.46	38.54	31.36	29.77	33.22
6	45.61	36.49	35.31	39.14	60.41	47.39	49.17	52.32
8	53.75	41.61	43.81	52.68	65.59	46.25	49.42	53.75
10	60.29	48.98	48.76	46.39				
12	47.51	21.32	24.90	27.91				
Mean	39.61	29.71	29.88		40.55	32.18	32.85	

CD ($P \leq 0.05$)

DOS days of storage

[a]Days of storage 1.634, treatments 1.069, interactions 2.83

[b]Days of storage 1.84, treatments 1.425, interactions 3.186

calcium treatment are supported by Siddiqui et al. (2004) in apple and Zhi et al. (2017) in peach during storage. The activities of cell wall degrading enzymes (Cellulase, PME and PG) got reduced by pretreatment of Ber fruits with both 1% and 2% $CaCl_2$ thus indicating calcium mediated delay in fruit softening and maintenance of integrity of cell wall in both the varieties.

Correlation analysis

Studies on correlation between firmness, cell wall components and its hydrolyzing enzymes in both the Ber varieties are presented in Table 4. The percentage increase in firmness in $CaCl_2$ treated fruits is positively correlated ("r" > 90%) with increase in cell wall components viz. cellulose, hemicellulose and pectin and it was negatively correlated (> 90%) with activities of cell wall degrading enzymes in both the varieties thus indicating that the maintenance of firmness by $CaCl_2$ treatment has positive correlation with cell wall stability. This is further substantiated by a significant positive correlation between calcium mediated retention of fruit firmness and increase in cell wall components and decrease in cell wall degrading enzyme activities.

Conclusion

The present results confirm that decrease in cell wall components viz. cellulose, hemicellulose and pectin in coordination with enhancement in the activities of the cell wall hydrolyzing enzymes throughout the storage of Ber fruits resulted in disassembly of cell wall and modification of its components and ultimately loss in the texture and

Table 4 Pearson correlation coefficients between calcium mediated changes (percent) in firmness, cell wall components and their hydrolyzing enzymes in Ber fruits

	Hemicellulose	Cellulose	Pectin	Poly-galacturonase	Cellulase	Pectin methylesterase	Firmness
Umran							
Hemicellulose	1	1.000**	1.000**	− 0.999*	− 0.999*	− 0.999*	0.940
Cellulose		1	1.000**	− 0.999*	− 0.999*	− 1.000**	0.944
Pectin			1	− 0.999*	− 0.999*	− 1.000*	0.943
Polygalacturonase				1	0.996	1.000*	− 0.955
Cellulase					1	0.997	− 0.925
Pectin methylesterase						1	− 0.952
Firmness							1
Kaithali							
Hemicellulose	1	0.997*	0.994	− 0.993	− 1.000**	− 1.000*	0.970
Cellulose		1	1.000*	− 0.999*	− 0.997*	− 0.995	0.985
Pectin			1	− 1.000**	− 0.995	− 0.992	0.990
Polygalacturonase				1	0.993	0.990	− 0.992
Cellulase					1	1.000*	− 0.970
Pectin methylesterase						1	− 0.964
Firmness							1

firmness of fruits. The more pronounced increase in the soft variety, Kaithali than in the firm Umran indicates that loss of firmness is the function of the modification of cell wall components catalyzed by various cell wall degrading enzymes. Pretreatment of fruits with calcium chloride could be effective in delaying the softening by maintaining the tissue structure and cell wall constituents and reducing the activities of the cell wall degrading enzymes.

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