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Effect of Hot Calcium Chloride and Acetic Acid Solutions or Hot Water Treatments on Inhibition of Total Microbial Counts and Browning in Lettuce Stem Cut During Cold Storage

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Abstract

Heads of iceberg lettuce (*Lactuca sativa* L. cv. Iceberg) were harvested at the proper stage of maturity from Kaha Experimental Farm, Qalubia Governorate, Egypt, during two successive seasons in 2015 and 2016. Good heads were transported immediately to the laboratory of Handling of Vegetable Crops Department Horticulture Research Institute at Giza. Egypt. All leaves were removed and stems were cut by a sharp knife into round slices (1 cm thickness × 2.5 diameter) then dipped for 1 min in the following solutions, hot $CaCl_2$3% or 5% at 50ºC, hot acetic acid 1% or 3% at 50ºC, $CaCl_2$3% or 5% at 20ºC, acetic acid 1% or 3% at 20ºC, hot water at 50ºC and distilled water (control) to study the effect of these treatments on inhibition butt discoloration and microbial count of the stem cut lettuce during storage at 2±1 °C and 95 % relative humidity (RH) for 10 days. Results indicated that all studied treatments reduced browning index, Polyphenol oxidase (PPO) activity, Phenylalanine ammonia-lyase (PAL) activity, had lower level of microbial load and maintaining total phenolic contents of the stem cut lettuce during cold storage comparing with untreated control. Stem cut lettuce dipped in hot Calcium Chloride 5% solution showed a positive effect on enzymes related to color maintenance. It reduced the activity of the browning related enzymes polyphenol oxidase (PPO) and Phenylalanine ammonia-lyase (PAL), maintained of total phenolic content, had the lowest total bacterial counts and inhibited browning on the cut surface of lettuce stem during storage at 2±1 °C and 95 % relative humidity for 10 days.

Keywords: Iceberg lettuce (*Lactuca sativa* L.), Hot Calcium Chloride and Acetic Acid, Hot Water, Microbial Counts, Browning.

Introduction

Butt discoloration (browning of the cut stem) is one of the main changes in lettuce (*Lactuca sativa* L. cv. Iceberg) during postharvest. Wounding iceberg lettuce stem tissue induce Phenylalanine ammonia-lyase (PAL) activity and the synthesis and accumulation of soluble phenolic components (caffeic acid derivatives). Oxidation of phenolic compounds to *o*-quinones, are action catalyzed by polyphenol oxidase (PPO). Quinones then polymerize to form dark brown, black or red polymers (Sapers and Hicks, 1989). This browning process decreases marketability of fruits and vegetables. The enzyme PPO was described as o-diphenol-oxygen oxidoreductase which is exclusively substrate specific for *o*-dihydroxy substrates. It has an optimum pH between 5–8 and an optimum temperature around 25–35 ºC (Fujita *et al*., 1991). It also has different conformations and native molecular weights in photosynthetic and vascular lettuce tissues (Heimdal *et al*., 1994).

Enzymatic browning (EB) does not occur in intact plant cells since phenolic compounds in cell vacuoles are separated from the PPO which is present in the cytoplasm. Once tissue is damaged by slicing, cutting or pulping, however, the formation of brown pigments occurs. The rate of EB in fruit and vegetables is governed by the active PPO and phenolic content in tissue, pH, temperature and O_2 availability within in the tissue (FAO, 2000). The main step in enzymatic browning is the oxidation of phenolic compounds by PPO in the presence of oxygen (Karrayan and Aydemir, 2001). Therefore, it is very important to prevent these reactions. Various techniques and mechanisms have been developed over the years for the control of the undesirable enzyme activities, these techniques attempt to eliminate one or more of the essential components (O_2, enzyme, copper or substrate) from the reaction (FAO, 2000). Among the compounds that have been shown to inhibit browning as postharvest treatment are calcium chloride Eleni and Theodoros (2011) acetic acid (Tomas-Barberan *et al.*, 1997) and heat shock treatment (Bakeer. 2016).

Calcium chloride has been tested as antibrowning agents. Calcium treatments is known as a potential postharvest approach used to maintain quality and to extend shelf life of fresh commodities because of its effects in controlling physiological disorders and delaying senescence in fresh commodities (Lester and Grusak, 1999). Supapvanich *et al.* (2012) found that hot $CaCl_2$ treatment also preserved nutritional quality of fresh-cut sweet leaf bush, especially antioxidant activity and biologically active compounds (total phenolics, total flavonoids, total ascorbic acid contents and antioxidant enzymes activity) during storage, which plays an important role as a health benefits for human beings.

Heat shock treatments, alone or combined with other agents, have also been used to prevent browning reactions and maintain texture in various vegetables and fruits (Murata *et al.*, 2004). Hot water treatments

alone have been shown to be an effective physical method for the control of a wide range of pathogens for storage rots (Palou *et al*., 2001). Besides reduction of storage decay, dipping fresh produce in hot water is believed to improve the quality of fruit for prolonged storage, a heat shock host response from hot water treatment has been found to weaken fungal growth by inducing host antifungal compounds involved in resistance (Fallik *et al*., 1996). Martın-Diana *et al.* (2006) reported that the use of heat-shock in combination with calcium lactate maintained objective and sensorial textural properties of fresh-cut lettuce during storage.

Acetic acid is a proven antimicrobial agent and a natural and safe food ingredient (Radi *et al*., 2010), also it is a valid candidate and effective in preventing postharvest fruit decay caused by *P. digitatum* and *P. italicum*. Acetic acid provided the highest protection against browning on the cut surface of lettuce cores (Tomas-Barberan *et al.*, 1997). Castaner *et al*. (1996) found that vinegar and 50 mL/L acetic acid solution inhibited browning on lettuce stem cuttings and could be used to prevent lettuce butt discoloration during cold storage and commercial handling.

Use of treatments where hot water and acetic acid are combined, so as to reduce both the acetic acid concentrations and water temperatures, deserves evaluation because this approach could conceivably reduce safety issues, improve the efficacy of the treatment compared with either acetic acid or hot water alone, and minimize injuries to the treated products. The synergistic effect of hot water treatment combined with low acetic acid concentrations to control the postharvest decay of apple fruit (Radi *et al.*, 2010) has been demonstrated. Organic acids (e.g. citric acid, tartaric acid, malic acid, sorbic acid, lactic acid, acetic acid) are known as weak acids having different inhibitory effects compared to strong acids. They acidify the cells´s interior by being lipophilic and penetrating the plasma membrane (Akbas and Olmez, 2007). The decline in intracellular pH results in inhibition of glycolysis and cell transport system (Rosa *et al.*, 2009).

Our objective was to study the effect of postharvest dipping in some antibrowning agent treatments on inhibition browning and microbial growth of lettuce cut stem during storage.

Material and Methods

Heads of iceberg lettuce (*Lactuca sativa* L. cv. Iceberg) were harvested at the proper stage of maturity from Kaha Experimental Farm, Qalubia Governorate, Egypt, during two successive seasons in 2015 and 2016. Good heads were transported immediately to the laboratory of Handling of Vegetable Crops Department Horticulture Research Institute at Giza. Egypt. All leaves were removed and stems were cut by a sharp knife into round slices (1 cm thickness × 2.5 diameter) (the first disk, which was the closest to the butt end, was discarded) then dipped for 1 min in the following solutions hot $CaCl_2$ 3% or 5% at 50ºC, hot acetic acid 1% or 3% at 50ºC, $CaCl_2$ 3% or 5% at 20ºC, acetic acid 1% or 3% at 20ºC, hot water at 50ºC and distilled water (control) . All samples were air dried and every five slices were packed in sealed polypropylene bags of size (15 cm × 20 cm and 30 µm thickness) to serve as one replicate. Fifteen replicates were prepared for each treatment. Samples were arranged in a complete randomized design and stored at 2±1 °C and 95 % relative humidity for 10 days. Measurements were recorded immediately after harvest and every 2 days interval to determine the following parameters.

Polyphenol oxidases (PPO) activity:

Polyphenol oxidases (PPO, E.C. 1.14.18.1) activity in iceberg lettuce extract, during the storage period was determined according to Pizzocaro *et al*. (1993).

Phenylalanine ammonia-lyase (PAL) activity:

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) activity was measured in the protein extract as previously described by Ke and Saltveit (1989) with slight modification by Campos-Vargas *et al*. (2005).

Total phenolic content:

Total phenolic contents were measured according to Singleton and Rossi, (1965).

Browning Index (BI):

The color of the lettuce slices stem disc was measured using a Minolta Chroma Meter (Model CR-155, Minolta Camera Co., Osaka, Japan), using the Hunter Lab Color Scale. Four lettuce slices stem were randomly selected from each sample and the color was measured at four equidistant points on each lettuce slices stem disc using an aperture diameter of 4 mm. lettuce slices stem disc color has been commonly measured using the L* value of the Hunter scale (Cliffe-Byrnes and Beirne, 2007). Also to changes in other parameters of the hunter scale (a* and b*) related to browning (Aguirre *et al*., 2008). In order to capture this variation in a single index that would be related to a turn towards brown color, browning index (BI) was calculated according to Bozkurt and Bayram (2006) and Ruangchakpet and Sajjaanantakul, (2007) using the following equation:

Browning Index (BI) = [100 (x - 0.31)] / 0.17, where x = (a* + 1.75L*) / (5.645L* + a* – 0.3012b*), L* value indicates lightness of the color, which range from 0 (dark) to 100 (white). The positive value of a* indicates red color, while negative value indicates green color. The positive value of b* indicates yellow color, while negative value indicates blue color.

Total microbial counts (TBC):

The population of total *microbial* counts (TMC) was determined by the method of Gonulalan *et al*., (2003).

All data were subjected to the statistical analysis according to the method described by Sendecor and Cochran (1980).

Results and Discussion

Polyphenol oxidase (PPO) activity:

Data in Table (1) show that there was considerable increase in PPO activity as the storage period was extended. These results are in agreement with those obtained by Shehata *et al.* (2012) who described how maximum PPO values appear after the shredding of lettuce during the later days of shelf-life. Also, data in the same table show that all antibrowning agents significantly affected PPO during storage compared with untreated control. Moreover, data cleared that, lettuce slices dipped in hot $CaCl_2$ 5% was the most effective in reducing PPO activity, following by hot $CaCl_2$ 3% or hot acetic acid 3 %, while the other antibrowning agents were less effective in this concern. Untreated control had the higher increase in the activity of PPO enzyme. These results were true in the two seasons and in agreement with Martın-Diana *et al.* (2006) for $CaCl_2$ and Radi *et al.* (2010) for hot acetic acid. Calcium had a significant inhibitory effect on PPO activity, resulting in decrease of the activity with increasing calcium concentration, the role of calcium might be to preserve membrane structure, and thereby keep PPO in its latent form, or to prevent cellular de compartmentalization and the mixing of soluble phenolic substrates, which are vacuolar, and membrane-bound PPO. In addition, PPO could be directly inhibited by $CaCl_2$ (Tomas-Barberan *et al.*, 1997). Dipping of Atemoya fruits with calcium chloride solution caused a significant decrease in PPO activity during storage, which was greater at 40 °C so that the temperature showed a synergistic effect (Torres *et al*., 2009).

The inhibitory mechanism of organic acids (acetic acids) is to maintain the pH well below that necessary for optimal PPO activity (McEvily *et al.*, 1992). The optimal pH range for lettuce PPO has been reported to be between 5 and 8 in vascular tissue (Heimdal *et al*., 1994), and 4.5 for whole lettuce (Fujita et al., 1991). However, the PPO activity was not totally suppressed at pH values below the optimum, i. e. 60% of the activity was detected when the pH was decreased to 3.5 (Heimdal *et al*., 1994).

Regarding the interaction between anti browning agents and storage periods, data showed that after 10 days of storage slices of lettuce dipped in hot solution of $CaCl_2$ 5% or 3% and hot acetic acid 3 % were reduced PPO activity with significant differences between them in both seasons.

Table 1. Effect of hot calcium chloride and acetic acid solutions or hot water treatments on Polyphenol oxidase (PPO) activity (Unit/g fresh weight) of lettuce stem cut during cold storage at 2 °c in 2015 and 2016 seasons.

Treatments	Storage period in days						
	0	2	4	6	8	10	M
				2015			
$CaCl_2$ 5% at 50 ºC	42.00	43.20	44.43	45.70	46.11	46.31	44.63
$CaCl_2$ 3% at 50 ºC	42.00	43.40	44.85	45.95	47.02	47.95	45.19
Acetic acid 3% at 50 ºC	42.00	43.44	45.05	46.72	48.45	50.24	45.98
Acetic acid 1% at 50 ºC	42.00	43.68	45.43	47.24	49.13	51.10	46.43
$CaCl_2$ 5%	42.00	43.91	45.90	47.99	50.17	52.45	47.07
$CaCl_2$ 3%	42.00	44.33	46.80	49.40	53.14	55.09	48.46
Acetic acid 3%	42.00	43.80	45.70	48.75	52.00	53.46	47.62
Acetic acid 1%	42.00	45.77	49.21	53.70	56.20	60.12	51.17
Hot water at 50 ºC	42.00	45.82	50.91	56.57	60.23	62.34	52.98
(Distilled water) Control	42.00	46.67	52.41	58.23	62.41	66.42	54.69
M	42.00	44.40	47.07	50.02	52.49	54.55	
				2016			
$CaCl_2$ 5% at 50 ºC	43.55	43.77	45.98	47.25	47.66	47.86	46.01
$CaCl_2$ 3% at 50 ºC	43.55	44.95	46.40	47.50	48.57	49.50	46.74
Acetic acid 3% at 50 ºC	43.55	44.89	46.60	48.27	50.00	51.79	47.52
Acetic acid 1% at 50 ºC	43.55	45.52	46.98	48.79	51.60	52.65	48.18
$CaCl_2$5%	43.55	45.00	47.45	49.54	51.72	54.00	48.55
$CaCl_2$3%	43.55	45.88	48.35	50.95	54.69	56.64	50.01
Acetic acid 3%	43.55	45.35	47.25	50.30	53.55	55.01	49.17
Acetic acid 1%	43.55	47.32	50.76	55.25	57.75	61.67	52.72
Hot water at 50 ºC	43.55	47.37	52.46	58.12	61.78	63.89	54.53
(Distilled water)Control	43.55	48.22	52.02	60.12	63.96	67.97	55.97
M	43.55	45.83	48.42	51.61	54.13	56.10	
LSD at .05 level		2016			2017		
Treatments(T)		0.38			0.33		
Storage periods(S)		1.37			1.44		
S *T		1.54			0.72		

Browning index (BI)

Browning index as well as white color measurement is good indicators for discoloration. Browning index formula considered the changes in a* (red and green color), b* (yellow and blue color) and L* (white color) which was more suitable for color changes measurements. The browning index of lettuce slices stem disc is related to change in color of lettuce slices stem disc. As the L* value decreased and a* and b* value increased, the browning index increased (Gupta and Bhat, 2016).

Storage time is important factors in BI deterioration of lettuce slices, though with the increase in storage period BI increased in both seasons (Table 2). Murata *et al*. (2004) found that stored cut lettuce gradually turns brown on the cut section after several days of storage, these results were true in the two seasons and in agreement with Tomas-Barberan *et al.* (1997) found that wounding iceberg lettuce stem tissue induces PAL activity and the synthesis and accumulation of soluble phenolic compounds (caffeic acid derivatives). Oxidation of these compounds to o-quinones by POP, and the polymerization of these quinones produces insoluble brown pigments.

Data also showed that all antibrowning agents reduced the BI compared to untreated control. Samples dipped in hot $CaCl_2$ 5% had significantly the lowest browning index throughout the whole storage time. This reduction was significant at each data point in both seasons followed by hot $CaCl_2$ 3% or hot acetic acid 3%. At the end of storage, a different browning index (BI) was observed in lettuce slices treated with different treatments with values ranged from 5.33 to 16.78 in the first season and 4.72 to 14.26 in the second season that was attributed to a generalized enzymatic browning in lettuce during storage. Lettuce slices dipped in hot $CaCl_2$ 5% gave the lowest value of BI while, the highest ones was obtained from untreated control.

Previous research has shown that increased levels of calcium within membranes can slow senescence and maintain the selective permeability of membranes (Ferguson, 1984). Martin-Diana *et al.* (2005) indicate that increased levels of calcium in lettuce slices stem disc treated with $CaCl_2$ may have decreased browning by increasing vacuolar membrane integrity, thereby reducing the opportunity for tyrosinase to react with its phenolic substrates. The effect of $CaCl_2$ in preventing browning must, therefore, be at the level of PPO. This enzyme is membrane-bound (Mayer, 1987), and is present in a latent form in lettuce tissue (Chazarra et al., 1996).When wounding occurs, membranes are degraded, and the fatty acids released can activate the latent PPO (Mayer 1987).

Table 2. Effect of hot calcium chloride and acetic acid solutions or hot water treatments on browning index of lettuce stem cut during cold storage at 2 °c in 2015 and 2016 seasons.

Treatments	Storage period in days						
	0	2	4	6	8	10	M
				2015			
$CaCl_2$ 5% at 50 ºC	0.51	1.50	2.74	3.68	5.19	5.33	3.16
$CaCl_2$ 3% at 50 ºC	0.51	1.11	2.51	4.10	6.80	7.11	3.69
Acetic acid 3% at 50 ºC	0.51	1.36	3.13	4.89	7.90	8.99	4.46
Acetic acid 1% at 50 ºC	0.51	1.11	2.97	4.76	8.76	9.88	4.67
$CaCl_2$ 5%	0.51	1.79	3.16	5.47	10.70	11.10	5.46
$CaCl_2$ 3%	0.51	1.61	3.25	6.14	10.95	11.78	5.71
Acetic acid 3%	0.51	1.94	4.38	6.90	11.20	12.75	6.28
Acetic acid 1%	0.51	2.03	3.86	7.37	14.19	15.64	7.27
Hot water at 50 ºC	0.51	2.51	3.95	7.89	15.04	15.98	7.65
(Distilled water) Control	0.51	2.78	5.99	10.88	15.95	16.78	8.82
M	0.51	1.77	3.59	6.21	10.67	11.53	
				2016			
$CaCl_2$ 5% at 50 ºC	0.32	1.13	1.64	2.80	4.36	4.72	2.49
$CaCl_2$ 3% at 50 ºC	0.32	0.83	1.51	3.12	5.71	5.97	2.91
Acetic acid 3% at 50 ºC	0.32	1.02	1.88	3.72	6.64	6.98	3.43
Acetic acid 1% at 50 ºC	0.32	0.83	1.78	3.62	7.36	7.47	3.56
$CaCl_2$ 5%	0.32	1.34	1.90	4.16	6.99	8.77	3.91
$CaCl_2$ 3%	0.32	1.21	1.95	4.67	9.20	9.81	4.53
Acetic acid 3%	0.32	1.46	2.63	5.24	10.14	10.70	5.08
Acetic acid 1%	0.32	1.52	2.32	5.60	11.92	12.09	5.63
Hot water at 50 ºC	0.32	1.88	2.37	6.00	12.63	12.88	6.01
(Distilled water)Control	0.32	2.09	3.59	8.27	13.40	14.26	6.99
M	0.32	1.33	2.16	4.72	8.83	9.36	
LSD at .05 level		2016			2017		
Treatments(T)		0.20			0.14		
Storage periods(S)		0.98			1.08		
S *T		0.67			0.44		

The role of calcium might be to preserve membrane structure, and thereby keep PPO in its latent form, or to prevent cellular decompartmentalization and the mixing of soluble phenolic substrates, which are vacuolar, and membrane-bound PPO. In addition, PPO could be directly inhibited by $CaCl_2$ (Tomas-Barberan *et al.*, 1997).

Wound-induced browning can be significantly reduced in iceberg lettuce by a short thermal stress (Loaiza-Velarde *et al.*, 1997).A few minutes exposure to 45ºC modified the physiological response (e.g. phenylpropanoid metabolism) of lettuce tissue to wounding (Loaiza-Velarde *et al.* 1997). Heat-shock treatments may reduce wound-induced increases in phenolic metabolism by interfering with the translation of wound-induced PAL mRNA into the PAL protein (Campos-Vargas *et al*., 2005).When PPO activity values were correlated with results of color analysis (appearance of browning), it was observed that lettuce treated at 50ºChad the lowest levels of browning (the lowest a* values) (Castaner *et al*., 1999). Martin-Diana *et al*. (2005) indicated that, the best treatment condition found in used a wash treatment solution of 1.5% calcium lactate at 50 ºC. From a quality point of view the use of high-temperature treatment (50ºC heat treatment) appears to inhibit browning reactions and improves texture. Torres *et al.* (2009) suggested that treated atemoya fruits with calcium treatment were significant reduction in PPO activity during storage, which was greater at 40°C so that temperature exhibited a synergic effect.

Castaner *et al.* (1996) indicated that, 50 mL/L acetic acid solutions could be very useful in preventing browning in lettuce cut stem during cold storage and commercial handling. The effect of browning inhibition by acetic acid and other acids had been suggested to be due to the decrease in pH which decreases the activity of PPO. However, here we show that, in addition of this possible role, acetic acid has a direct effect on phenolic metabolism. It inhibits PAL activity and thereby prevents the formation of caffeic acid derivatives, while inducing the accumulation of other metabolites (Tomas-Barberan *et al.*, 1997).

phenylalanine ammonia-lyase (PAL) activity:

Stored cut lettuce gradually turns brown on the cut section after several days of storage, because cutting induces phenylalanine ammonia-lyase (PAL) activity, the biosynthesis of polyphenol is promoted, and the polyphenols are oxidized by polyphenol oxidase (Murata *et al.*,2004).

Data in Table (3) showed that phenylalanine ammonia-lyase (PAL) activity was increased with the prolongation of storage period, these results were true in the two seasons and in agreement with Tomas-Barberan *et al.* (1997) and Loaiza-Velarde and Saltveit (2001) found that preparation of lettuce stem disks induced PAL activity which started to increase after 12-24 h at 5 °C and reached maximal activity at 48 h.

Concerning the effect of antibrowning agents, data revealed that all treatments were effective in maintaining lower PAL activity during storage compared with untreated control. Moreover, lettuce slices treated with hot $CaCl_2$ 5% or hot $CaCl_2$ 3% and hot acetic acid 3% were the most effective treatments in reducing the increase of PAL activity. These results are in agreement with Tomas-Barberan *et al.* (1997) for $CaCl_2$ and acetic acid and Campos-Vargas *et al.* (2005) for hot water.

Calcium treatment decreased PAL activity, in agreement with previous reports (Ke and Saltveit, 1986), but it had only a slight effect on the accumulation of phenolic compounds, which were produced and accumulated to levels similar to those found in the control. The effect of CaCl2 in preventing browning must, therefore, be at the level of PPO. This enzyme is membrane-bound (Mayer, 1987), and is present in a latent form in lettuce tissue (Chazarra et al., 1996). When wounding occurs, membranes are degraded, and the fatty acids released can activate the latent PPO (Mayer 1987).

The effect of browning inhibition by acetic acid had been suggested to be due to the decrease in pH which decreases the activity of PPO. However, here we show that, in addition of this possible role, acetic acid has a direct effect on phenolic metabolism. It inhibits PAL activity and thereby prevents the formation of caffeic acid derivatives, while inducing the accumulation of other metabolites Tomas-Barberan et al. (1997).

Wound-induced browning can be significantly reduced in iceberg lettuce by a short thermal stress (Loaiza-Velarde et al., 1997). A heat shock of 45°C for 90 s effectively prevents the synthesis of PAL by wounded lettuce leaf tissue and its subsequent browning. Inhibition of PAL synthesis appears to result from a redirecting of protein synthesis away from wound-induced proteins (e.g., PAL) to the synthesis of hsps. The heat shock does not act through interfering with the wound signal since it is effective when administered either 4 h before or 4 h after wounding. The heat shock effect was so persistent that fresh-cut iceberg lettuce did not show any browning even after being held for 15 days in air at 5°C Saltveit (2000).

Heat shock inhibits the increase in wound-induced PAL activity by inhibiting the accumulation of PAL proteins (and thereby increased enzyme activity) either by preventing the translation or accelerating the turnover of PAL proteins Campos-Vargas et al. (2005). The heat-shock treatment not only reduced the wound-induced increase in PAL activity, but it also helped maintain the four measured attributes of lettuce quality near their control, nonwounded levels Loaiza-Velarde and Saltveit (2001). It is clear that heat shock treatments cause profound and interconnected changes in the 'normal'

physiology of plants in order to produce increased tolerance to other stress, there also appears to be a hierarchy in the tissue's response to stresses, such that the response to some stresses (e.g. heat shock) takes precedence over the response to other stresses (e. g. wounding), additional research is needed to differentiate among the ways by which heat shock alters the synthesis of various induced proteins Campos-Vargas et al. (2005).

For the interaction between antibrowning agents and storage period (Table 3), After 10 days of storage, results indicated that lettuce stem disks dipped in hot $CaCl_2$ 5% had significantly lower values of PAL activity compared with other treatments, followed by hot $CaCl_2$ 3%, hot acetic acid 3%. These results were true in the two seasons.

Table 3. Effect of hot calcium chloride and acetic acid solutions or hot water treatments on phenylalanine ammonia-lyase (Unit/g fresh weight) activity of lettuce stem cut during cold storage at 2 °c in 2015 and 2016 seasons.

Treatments	Storage period in days						
	0	2	4	6	8	10	M
				2015			
$CaCl_2$ 5% at 50 ºC	0.33	0.43	0.54	0.65	1.11	1.15	0.70
$CaCl_2$ 3% at 50 ºC	0.33	0.59	0.70	0.95	1.29	1.31	0.86
Acetic acid 3% at 50 ºC	0.33	0.51	0.80	1.03	1.39	1.43	0.92
Acetic acid 1% at 50 ºC	0.33	0.76	0.91	1.10	1.45	1.57	1.02
$CaCl_2$ 5%	0.33	0.64	0.87	1.23	1.63	1.65	1.06
$CaCl_2$3%	0.33	0.82	0.98	1.43	1.70	1.72	1.16
Acetic acid 3%	0.33	0.87	1.13	1.70	1.77	1.79	1.27
Acetic acid 1%	0.33	0.84	1.03	1.48	1.83	1.94	1.24
Hot water at 50 ºC	0.33	0.90	1.28	1.80	1.97	2.12	1.40
(Distilled water) Control	0.33	1.40	1.70	1.97	2.20	2.27	1.65
M	0.33	0.78	0.99	1.33	1.63	1.70	
				2016			
$CaCl_2$ 5% at 50 ºC	0.50	0.55	0.65	0.87	0.89	0.89	0.73
$CaCl_2$ 3% at 50 ºC	0.50	0.66	0.77	1.05	0.95	0.98	0.82
Acetic acid 3% at 50 ºC	0.50	0.57	0.87	1.17	1.05	1.09	0.88
Acetic acid 1% at 50 ºC	0.50	0.81	1.01	1.20	1.28	1.32	1.02
$CaCl_2$ 5%	0.50	0.71	1.01	1.33	1.35	1.52	1.07
$CaCl_2$ 3%	0.50	0.89	1.13	1.55	1.56	1.68	1.22
Acetic acid 3%	0.50	0.87	1.06	1.53	1.47	1.59	1.17
Acetic acid 1%	0.50	0.91	1.23	1.68	1.85	1.90	1.35
Hot water at 50 ºC	0.50	0.97	1.38	1.90	1.91	1.95	1.44
(Distilled water)Control	0.50	1.53	1.90	1.95	2.04	2.35	1.71
M	0.50	0.85	1.10	1.42	1.44	1.53	
LSD at .05 level		2016			2017		
Treatments(T)		0.09			0.04		
Storage periods(S)		0.11			0.17		
S *T		0.15			0.07		

Total phenolic content:

Data in Table (4) showed that total phenolic was decreased with the prolongation of storage period. The decrease in phenolic content may be due to the oxidation of PPO enzyme to give the colored quinones and quercetin was oxidized directly by PPO (Queiroz *et al.*, 2008). Moreover, Robards *et al.* (1999) found that phenolic compounds have a significant role oxidation process as antioxidants and as substrates in browning reactions.

Concerning the effect of antibrowning agents, data indicated that all treatments were effective in maintaining total phenolic contents during storage compared with untreated control. Moreover, lettuce slices dipped in hot $CaCl_2$ 5% or hot $CaCl_2$ 3% and hot acetic acid 3% were the most effective treatments in reducing the loss of total phenolic contents with significant differences between them in the two seasons, however, the other treatments were less effective in this concern. The lowest value was obtained from untreated control. These results were true in the two seasons and in agreement with Tomas-Barberan *et al. (*1997) for calcium chloride and acetic acid and Wulfkuehler *et al.* (2013) for hot water treatment. For the interaction between antibrowning agent treatments and storage period, After 10 days of storage, results indicated that lettuce slices dipped in hot $CaCl_2$ 5% had significantly higher values of total phenolic compared with other treatments, followed by hot $CaCl_2$ 3%, hot acetic acid 3% with significant differences between them. These results were true in the two seasons.

Table 4. Effect of hot calcium chloride and acetic acid solutions or hot water treatments on Total phenolic content (Unit/g fresh weight) of lettuce stem cut during cold storage at 2 °c in 2015 and 2016 seasons.

Treatments	Storage period in days						
	0	2	4	6	8	10	M
				2015			
$CaCl_2$ 5% at 50 °C	17.52	17.30	17.08	17.00	16.61	16.46	17.00
$CaCl_2$ 3% at 50 °C	17.52	17.10	17.15	16.06	15.53	15.40	16.46
Acetic acid 3% at 50 °C	17.52	17.00	16.11	16.01	15.31	15.21	16.19
Acetic acid 1% at 50 °C	17.52	16.52	16.14	15.47	14.81	14.71	15.86
$CaCl_2$ 5%	17.52	16.24	16.00	15.32	14.10	13.87	15.51
$CaCl_2$ 3%	17.52	16.52	15.71	14.70	13.79	13.35	15.27
Acetic acid 3%	17.52	16.52	15.64	14.66	13.55	12.97	15.14
Acetic acid 1%	17.52	16.24	15.51	14.51	13.44	12.88	15.02
Hot water at 50 °C	17.52	16.02	15.47	14.32	13.24	12.70	14.88
(Distilled water) Control	17.52	15.64	15.22	14.00	13.03	12.00	14.57
M	17.52	16.51	16.00	15.21	14.34	13.96	
				2016			
$CaCl_2$ 5% at 50 °C	15.95	15.82	15.74	15.61	14.98	14.63	15.46
$CaCl_2$ 3% at 50 °C	15.95	15.53	15.34	15.00	14.35	13.95	15.02
Acetic acid 3% at 50 °C	15.95	15.41	15.24	14.92	14.00	13.61	14.86
Acetic acid 1% at 50 °C	15.95	14.45	14.32	14.00	13.52	13.21	14.24
$CaCl_2$ 5%	15.95	14.15	13.51	13.22	13.11	12.34	13.71
$CaCl_2$ 3%	15.95	14.12	13.95	13.51	13.07	12.75	13.89
Acetic acid 3%	15.95	14.07	13.58	13.14	12.26	12.50	13.58
Acetic acid 1%	15.95	13.77	13.51	13.11	13.00	12.23	13.60
Hot water at 50 °C	15.95	13.51	13.21	12.54	12.41	12.04	13.28
(Distilled water)Control	15.95	13.14	12.87	12.54	12.11	11.87	13.08
M	15.95	14.40	14.13	13.76	13.28	12.91	
LSD at .05 level		2016			2017		
Treatments(T)		0.09			0.11		
Storage periods(S)		0.14			0.21		
S *T		0.21			0.13		

Total bacterial content (TBC):

Table (5) presents growth of total bacteria counts (TBC) (expressed as log cfu/g^{-1}) of lettuce slices stem disks during 10 days of storage. Gradual growth of microorganisms was seen during storage in all samples. TBC of lettuce slices stem disc are usually due to the growth of *Pseudomonas* bacteria Akbas and Olmez (2007). As these bacteria grow, they break down the lettuce fibers which soften the lettuce and leads to enzymatic browning (Campos-Vargas *et al*., 2005). There were significant different in TBC between all treatments and untreated control in the two seasons. The highest amount of TBC was observed in control samples. Samples hot $CaCl_2$ 5%were found to be significantly effective in delaying TBC in lettuce stem disks during storage, followed by hot $CaCl_2$ 3% or hot acetic acid 3% with significant deferent between them in the two seasons. $CaCl_2$ at 5% or 3%, acetic acid at 3% or 1% and hot water at 50 °C treatments were less effective in this concern.

Concerning the interaction between antibrowning agents and storage periods, data revealed that there was significant difference in the bacterial number between the control and other treatments lettuce during 4 days (Table. 5), after 10 days of storage, data showed that, the bacterial number in all treatments was significantly lower than in the untreated control. However, the amount of TBC in the hot $CaCl_2$ 5% and hot $CaCl_2$ 3% treatments were 3.70 and 4.29 log cfu/g^{-1} respectively (average of the two seasons), while in untreated control was 7.86 log cfu/g^{-1} (average of the two seasons). These results are in agreement with Murata *et al*. (2004).

The mechanism by which increased tissue Ca reduces decay and maintains firmness is hypothesized to be related to Ca ions in the cell wall (Demarty *et al*., 1984). Cell wall pectins are composed primarily of four-linked galacturonosyl residues, with varying amounts of two-linked rhamnosyl residues interspersed in the chain (Preston, 1979). The stability of the cell wall may be related to the cooperative binding of polygalacturonate chains with Ca ions (Grant *et al*., 1973 and Knee, 1978), making the cell wall of the fruit less accessible to enzymes that cause softening or to cell wall degrading enzymes produced by fungal pathogens. Increasing the Ca content of apple cell walls has been shown to inhibit maceration by polygalacturonase produced by *P. expansum* (Conway *et al*., 1988).

It is well known that many pathogenic and spoilage microorganisms cannot grow at low pH values, organic acids have antimicrobial properties by decreasing the pH of the applied solution, the

dissociation of hydrogen ions causes reduction in the internal cellular pH of the organism (Tirpanalan *et al*., 2011). Disruption in the ability of the cell maintaining the pH homeostasis results in disruption of membrane permeability and substrate transport (Jongen. 2005). Organic acids (acetic acid) are known as weak acids having different inhibitory effects compared to strong acids, They acidify the cell's interior by being lipophilic and penetrating the plasma membrane, The decline in intracellular pH results in inhibition of glycolysis and cell transport system (Rosa *et al*., 2009).

The mechanism by which heat treatment reduces decay and maintains firmness through effects on the cell wall has not been explained satisfactorily. Heat inactivation of synthesis of pectin-degrading enzymes was considered a possible mechanism, and analysis of 'Golden Delicious' apple fruit following storage showed significantly lower levels of soluble pectin in juice of heat-treated fruit than controls (Shao *et al*., 2007). Heat treatment may inhibit protein synthesis required for cell wall degradation and ethylene synthesis (Lurie and Klein, 1990).

Ca probably resulted from increased resistance of tissue to bacterial infection rather than to a bactericidal action, Ca enhanced tissue resistance to fungal attack by stabilizing or strengthening cell walls thereby making them more resistant to pectolytic enzymes produced by fungi (Izumi and Watada.1994). The inhibitory effect of some Ca salts on microbial growth has been related to cell wall stability by ionic Ca and polygalacturonate chains, since Ca increases the rigidity of the cell wall and middle lamella and therefore the resistance to fungal enzymes, decreasing softening and cell wall degradation (Silveira et al., 2011). Aguayo et al. (2008) also reported that CaCl2 reduced microbial counts by 2 log units.

Li et al. (2001) reported that dipping fresh-cut iceberg lettuce for 1.5 min in water at 50°C significantly reduced the initial population of psychotropic and mesophilic aerobic microflora by l.73 - l.96 cfu/g-1 as compared to untreated samples.

Table 5. Effect of hot calcium chloride and acetic acid solutions or hot water treatments on total bacterial content (cfu/g^{-1}) of lettuce stem cut during cold storage at 2 °c in 2015 and 2016 seasons.

Treatments	Storage period in days						
	0	2	4	6	8	10	M
				2015			
$CaCl_2$ 5% at 50 ºC	0.30	1.33	2.18	3.00	3.81	3.98	2.43
$CaCl_2$ 3% at 50 ºC	0.30	1.29	2.25	3.10	4.11	4.33	2.56
Acetic acid 3% at 50 ºC	0.40	1.38	2.33	3.15	4.22	4.80	2.71
Acetic acid 1% at 50 ºC	0.45	1.44	2.49	3.20	4.31	4.95	2.81
$CaCl_2$ 5%	0.60	1.59	2.61	3.66	4.74	5.15	3.06
$CaCl_2$ 3%	0.74	1.70	2.68	3.49	4.71	5.33	3.11
Acetic acid 3%	0.65	1.62	2.71	3.54	4.64	5.41	3.10
Acetic acid 1%	0.72	1.75	2.80	3.60	4.75	5.45	3.18
Hot water at 50 ºC	0.95	2.00	2.95	4.12	5.12	6.88	3.67
(Distilled water) Control	3.40	4.35	5.20	6.00	7.10	7.90	5.66
M	0.85	1.85	2.82	3.69	4.75	5.42	
				2016			
$CaCl_2$ 5% at 50 ºC	0.20	1.20	1.42	1.78	2.66	3.41	1.78
$CaCl_2$ 3% at 50 ºC	0.22	1.21	1.98	2.87	3.67	4.25	2.37
Acetic acid 3% at 50 ºC	0.32	1.30	2.25	3.07	4.14	4.72	2.64
Acetic acid 1% at 50 ºC	0.37	1.37	2.40	3.11	4.25	4.87	2.73
$CaCl_2$ 5%	0.52	1.51	2.53	3.58	4.55	5.11	2.97
$CaCl_2$ 3%	0.65	1.62	2.60	3.41	4.63	5.25	3.03
Acetic acid 3%	0.57	1.54	2.63	3.46	4.56	5.33	3.02
Acetic acid 1%	0.64	1.67	2.72	3.52	4.67	5.37	3.10
Hot water at 50 ºC	1.85	3.92	4.77	4.90	5.66	7.04	4.69
(Distilled water)Control	3.32	4.27	5.12	5.92	7.02	7.82	5.58
M	0.87	1.96	2.84	3.56	4.58	5.32	
LSD at .05 level		2016			2017		
Treatments(T)		0.11			0.22		
Storage periods(S)		0.54			0.41		
S *T		0.34			0.33		

Conclusions

For the previous results, it could be concluded that hot $CaCl_2$5% at 50ºC solutions could be very useful in preventing browning in lettuce cut stem, which reduced the activity of PPO and PAL enzymes, maintained total phenolic content and had the lowest total bacterial count and browning index during storage at 2±1 °C and 95 % relative humidity for 10 days.

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تأثير محاليل كلوريد الكالسيوم وحمض الخليك الساخن أو الماء الساخن على تثبيط الحمل الميكروبي والتلون البنى في منطقة سطح القطع للخس الكروى أثناء التخزين المبرد

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تم جمع رؤوس الخس الكروى صنف ايس بيرج فى مرحلة النضج المناسبة من محطة التجارب بقها محافظة القليوبية خلال موسمى 2015،2016 . تم نقل الرؤوس السليمة الى معمل بحوث تداول الخضر معهد بحوث البساتين بالجيزة حيث تم إزالة كل الاوراق وتقطيع السيقان الى شرائح مستديرة (سمك 1 سم ، قطر 2.5 سم) ثم غمرها فى المحاليل التالية ، كلوريد كالسيوم ساخن على درجة 50°م بتركيز 3 و 5 % و حامض خليك ساخن 50°م بتركيز 1 و 3 % و كلوريد كالسيوم بتركيز 3 و 5 % على درجة 20°م و حامض خليك بتركيز 1 و 3 % على درجة 20°م وماء ساخن على درجة 50°م بجانب الغمر فى الماء المقطر (كنترول) لدراسة تأثير هذه المعاملات على تثبيط التلون البنى والحمل الميكروبى لشرائح ساق الخس خلال التخزين على درجة 2± 1 °م ورطبة نسبية 95% لمدة 10 أيام . أوضحت النتائج أن كل المعاملات المستخدمة أدت الى تقليل التلون البنى ونشاط كلا من أنزيمى البولى فينول اكسيديز وفنيل الانين امينوليز كما أعطت أقل مستوى من الحمل الميكروبى مع الحفاظ على المحتوى من المركبات الفينولية لشرائح ساق الخس وذلك مقارنتا بالمعاملة كنترول. المعاملة بمحلول كلوريد كالسيوم ساخن على درجة 50°م بتركيز 5 % أعطت تأثير إجابى على الأنزيمات المتعلقة بالاحتفاظ باللون حيث أدت الى تقليل نشاط أنزيمى البولى فينول اكسيديز وفنيل الانين امينوليز مع الاحتفاظ بتركيز الفينولات مرتفعاً ، كما أعطت أقل عدد من الحمل الميكروبى وتثبيط التلون البنى على السطح المقطوع من الساق وذلك خلال التخزين على درجة 2± 1 °م ورطبة نسبية 95% لمدة 10 أيام.