Egypt. J. Hort. **Vol. 42,** No. 1, pp. 1 - 15 (2015)

The Role of Protein Contents and Enzyme Activity on Creasing of Washington Navel Orange Fruits

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CREASING is one of the main physiological disorders of navel orange fruit representing a high reduction percentage of fresh fruit. Creasing incidence, its possible relationship with the role of protein and enzyme activities were approached. A research study was conducted for two consecutive seasons to monitor and evaluate the protein and enzyme activities in creased and non-creased fruits of Washington navel orange in Delta, Egypt. Further parameters such as fruit physical and chemical characteristics were measured as well as the percentage of creased fruits as affected in three dates *i.e.* mid Jan., first of Feb. and first of March and two tree geographical directions, *i.e.* north and south. The obtained data revealed that creasing percentage was increased progressively with fruit aging, and creasing incidence was relatively influenced by geographical direction (more pronounced in fruits of northern tree periphery). Meanwhile, the protein banding patterns of albedo and flavedo total proteins exhibit the association between some particular protein types and the changing in citrus peel tissue from healthy to crease. Moreover, the higher amount of PG-ase release was tended to be closely related to albedo taken of creased fruits compared to non creased ones.

Keywords: Washington navel orange, Creasing, PG-ase activity, Protein banding patterns.

A profitable citrus industry is based on the optimum fruit production and quality. However, citrus fruits can face serious physiological disorders such as creasing, splitting and more. Creasing is a set of stressful environmental factors affecting on citrus genotypes - it is a recurrent problem in navel oranges - which leads to emersion the creased tissue. It is a peel-related problem which consists of small random cracks in the albedo tissue corresponding to sunken grooves on the fruit surface (Erickson, 1968). The degree of creasing incidence varied according to the citrus varieties (Zahoor and Zora, 2015). It is, also, known as a pre-harvest disorder as a result of the albedo breakdown initiating creases and/or irregular furrows in the exterior fruit rind surface. Sever creases can cause rind splitting during the harvesting, handling and transportation resulting in serious economic loss causing individual orchard losses often exceed 40 % based on the different creasing incidence (Gilfillan *et al.*, 1980).

Although no definite causes of creasing have been established, several factors have been associated with that disorder. Fruit position, climate, rind thickness, fruit size, tree heredity, irrigation, nutrition and rootstock (Monselise *et al*., 1976; Holtzhausen, 1982 and Treeby *et al*., 1995) have been reported.

Generally, the gene–environment interaction is an explicit factor for characters appearance. A norm of reaction is a graph that shows the relationship between genes and environmental factors when phenotypic differences are continuous (Krebs, 2012). Moreover, plants are unable to express their full genetic potential for production when subjected to stressful environments (Ziegler, 1990). It is also well documented that various environmental stresses caused important modification in gene expression in plants (Vierling, 1991). Such modification may lead to the accumulation or depletion of certain metabolites, alterations of the behavior of many enzymes and synthesis of new sets of proteins that are specific to the particular stress types (Jacobsen *et al*., 1986). Consequently, it has been shown that the different environmental stresses induce the synthesis of new proteins in plants, which possibly provides evolutionary value to the plants for survival under adverse environmental situations (Ericson & Alfinito, 1984, Hurkman & Tanaka, 1987 and Singh *et al*., 1987).

Additionally, Li *et al*. (2009) reported that the loss of pectin and cellulose in the albedo of creased orange fruit-rind cell walls is associated with the comparative changes in cell wall components and the activities of certain degrading enzymes such as PE, PG, cellulase and the expression of α–expansin genes. However, Jones and Embleton (1967) mentioned that, creasing of orange fruit was very variable among trees in the orchard, and became progressively more severe as the fruit aged. Total pectin (TP), water-soluble pectin content (WP) and pectinesterase (PE) in creasing fruits were higher than in normal fruits, while hydrochloric acid soluble pectin (HP) was higher in normal orange fruits. Chen, *et al*., 2005 and Basharat *et al*., 2014).

Although the above mentioned authors showed some contributing factors however none went further to explain the physiological basis of creasing development.

The objective of this two-year study on Washington navel orange (*Citrus sciences* L. Osbek) was to provide further physiological development understanding *i.e.* role of protein and the enzyme activities of the creasing disorder.

Materials and Methods

Thirty trees of 25-year-old Washington navel orange [*Citrus sciences* (L.) Osbek] grafted on sour orange rootstock grown in Delta, Egypt were randomly selected. The trees were labeled and divided into three replicates 10 trees per each. The north and south directions of each tree were labeled and monitored

during the growing seasons of 2009/2010 & 2010/2011. The following determinations were carried out:

Protein assay

Electrophoretic detection of protein by sodium dodecyle sulphate, polyacrylamide gel electrophoresis (SDS-PAGE):

Forty five fruits were selected and harvested in the first of March, then categorized into three categories, and 15 fruits per each category i.e. (Sever creased, Intermediate and healthy) were collected and prepared. Albedo and flavedo tissues from each category were separated chopped and freeze. Approximately 1 (g) of freeze tissues from each category was ground and crushed in mortar and pestle in liquid nitrogen till fully homogenized. A sample, then, transferred into 1 (ml) Eppendorf tube brought to 200 µl with extraction buffer (50 m M tris-HCl buffer, pH 6.8, glycerol 10 % w/v, ascorbic acid 0.1%, cycteine hydrochloride 0.1 w/v). The centrifugation at 18000 rpm for 30 mints was carried out to remove debris.

The protein content in supernatant was estimated according to the method of Bradford (1976) by using bovine serum albumin as a standard protein. Protein content was adjusted to 2 mg / ml per sample.

Separation of citrus peel proteins on the basis of molecular weight were analyzed by Sodium Dodecyle Sulphate, Polyacrylamide Gel Electrophoresis (SDS-PAGE) method as adapted by Laemmili (1970) with slight modifications to use in the present study. The modification, was reduced TEMED from 30 µl to 25 µl and also APS was reduced from 1.5 ml to 1.3 ml. The separation gel (12% W/V) and staking gel (4 % W/V) were used. At the end of the run the gel was stained according to silver staining method for protein described by Sammons *et al*. (1981).

This method of staining is sensitive and detects as little as 2 ng of protein in a single band. When the protein bands can be visually seen, data were recorded by photograph. Protein finger print characterizations of different citrus peel tissue (albedo and flavedo) were determined by scanning of the total citrus peel protein electrophoretic gel, using a computer technique.

The number of discrete protein bands and its molecular weight were determined by comparison with a medium molecular weight protein standard marker provided by Ferments.

Determination of PG-ase activity

PG-ase activity was determined in three categories of albedo tissues from (healthy fruit, non creased part of creased fruit and creased part of creased fruit) by measuring reducing groups release from albedo. In this method, the albedo samples were frozen, homogenized and centrifuged for 20 min. at 8000 rpm (Maud *et al*., 2005), then pellet was suspended in distilled water at pH 3, centrifuged at 12000 rpm and repeated until no reducing sugars were detected in

washings using the DNS method (Chaplin and Kennedy, 1997). Moreover, PG-ase release from peel samples was done after Ali & Brady (1992). Finally, the determination of PG-ase activity was done by measuring the amount of galacturonic acid (as reducing groups) using the 3,5 dinitrosalicylic acid method of Chaplin and Kennedy (1997).

Creasing incidence percentage

Fruit samples were monitored at three dates i.e. first of Jan., mid of Feb. and first of March each season. The fruits from south and north direction of each replicate were checked, counted and then classified into two categories i.e. creased and non-creased fruits. The creasing incidence percentages were calculated according to the following equation:

Creasing % = (Creased fruits/Total fruit number per each direction) x 100

Fruit physical properties

In the first of March, ten fruits from each category *i.e.* creased and non-creased were selected, harvested and the following parameters were measured, fruit weight (g), fruit volume (cm^3), fruit height (cm), fruit diameter (cm), fruit shape index (height /diameter),

Fruit chemical properties

The same fruits sample was used to determine the following fruit chemical properties, fruit TSS (%) using Carl Zeiss hand refractometer, the percentage of juice acidity expressed as (%) of citric acid, the TSS /acid ratio and vitamin C as ascorbic acid (mg/100 ml fruit juice weight) according to A.O.A.C. (1990).

Statistical analysis

Data obtained were statistically analyzed according to Snedecor and Cochran (1980). The means, standard deviations and an analysis of variance of three replicates were estimated using MSTAT-C software (Freed & Scott, 1986) while the comparison of mean effects was based on least significant difference (LSD) multiple-comparison tests. Significant differences were considered at $P < 0.05$. The correlation and linear trend was also used for certain data.

Results and Discussions

Protein assay

The protein banding pattern (protein finger print and protein profile) of albedo and flavedo total proteins and the analysis of SDS-PAGE showed total of 17 bands with molecular weight ranged from 15 to 69 kDa are presented in Photo 1 and Table 1. The three categories of tissues *i.e.* creased tissue (CT), Intermediate tissue – healthy tissue from simply creased fruit- (IT) and Healthy tissue (HT) were varied in both number of protein bands which were 16, 17 and 15 bands in albedo tissue and 11, 15 and 12 in flavedo, respectively. This variation clearly showed the correlation between the fettle of tissue and type of proteins.

It is noticed that the proteins of molecular weight 37 and 51 kDa appeared only in HT, the same trend was noticed in IT by 69 kDa protein and CT by 25, 26, 31, 32 and 35 kDa proteins. Data in hand strongly suggest that the two proteins (51 and 37 kDa) are playing a very important role to keep the tissue healthy and so they disappeared in IT and CT which mean, that the responsible genes for producing those two proteins (37 and 51 kDa) were switched off and the production of those proteins was stopped. This step allows the other genes starting work to transfer the albedo cells from healthy to creased ones going through intermediate phase by generating another proteins 69 in IT and (25, 26, 31, 32 and 35 kDa) in CT. These proteins in CT seemed to be strongly related to the incidence of creasing in citrus fruits. In this respect, it is important to point to the intermediate phase which had the highest number of protein bands (17) indicating that the tissue cells in this phase were slightly more active than the healthy or creased ones.

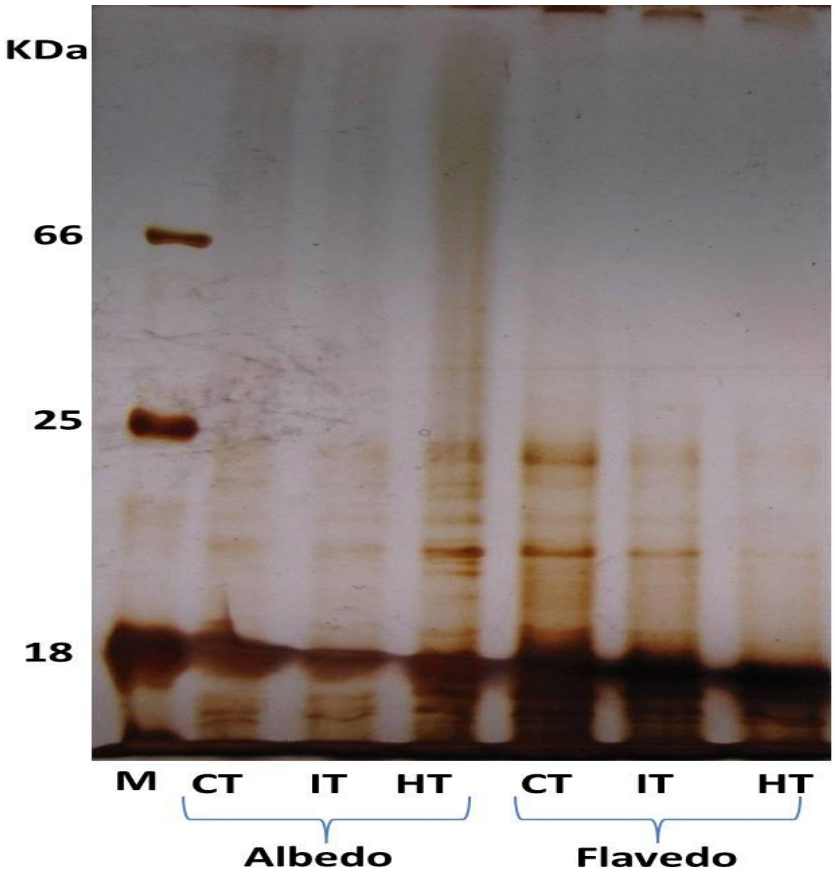

Photo 1. SDS-PAGE of citrus peel total proteins .

TABLE 1. The molecular mass of protein bands and percentage of protein amount in band of citrus peel protein profiles.

Protein (%) in bands						
	Albedo			Flavedo		
Protein mass (Kda)	**CT**	**IT**	**HT**	**CT**	**IT**	**HT**
69	-	0.84	-	-	-	-
68	-	-	-	-	0.46	-
51	-	-	10.87	-	-	-
38	-	-	-	-	1.46	-
37	-	-	9.40	-	-	-
35	4.08	-	-	-	-	-
34	-	-	-	-	-	11.22
32	1.05	-	-	-	-	-
31	2.19	-	-	-	-	-
26	5.30	-	-	-	-	-
25	10.33	-	-	-	7.51	5.46
24	-	12.4^{3}	16.96	31.13	23.31^{2}	9.24
23	6.85^{2}	8.81	7.16^{2}	5.29	5.57^{2}	10.72
22	5.15	8.21	5.11	5.10	5.64	-
21	8.48	20.70^{2}	7.04	7.17	9.25	27.50
20	3.26	5.24	3.58	6.05^{2}	7.51^{2}	5.09^{2}
19	9.99	10.02^{3}	5.15	8.08^{2}	1.47	3.78
18	29.75	19.40	19.49^{2}	3.76	6.22	0.88
17	-	-	-	32.38	26.11	19.83
16	10.93^{3}	11.94^{3}	7.21^{2}	-	-	2.97
15	2.65	3.00	8.05^{2}	1.02	4.89	3.31
Total bands	16	17	15	11	15	12

CT : Creased Tissue
IT : Intermediate Tissue (tissue from simply creased fruit)
HT : Healthy Tissue
$( )^{n}$: The number of protein bands which are similar in kDa molecular weight

In another word, the healthy and the creased tissue cells tended to be more stable. Moreover, one discrete band with highest molecular weight (69 kDa) appeared in this phase and disappeared in the next creased phase. In general, the same performance was noticed in HT and IT of flavedo with number of bands 12, 15 and 4 discrete bands with a molecular weight (16, 34 kDa) and (38, 68 kDa) for HT and IT, respectively. But this performance was differing in CT proteins banding pattern, the number of bands was 11 with no appearance of distinguishable bands.

These observations are logically acceptable because that the main physiological process occurred in albedo tissue, so that creasing is known as albedo breakdown, (Holtzhausen, 1982).

Furthermore, the dendrogram of clustering analysis (Fig.1) exhibit the similarity of protein bands. The highest percentage was between CT and IT proteins and it was (94.8 and 99.31%) in both albedo and felavedo tissue, respectively.

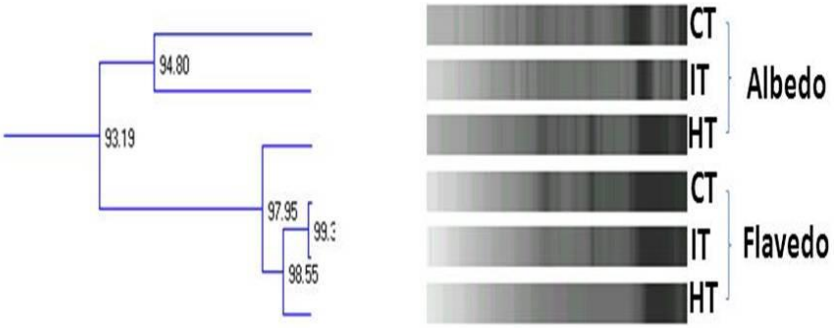

Fig. 1. Protein clustering analysis dendrogram of creased tissue (CT), intermediate tissue (tissue from simply creased fruit) (IT) and the healthy tissue (HT) of Washington navel orange fruits

Finally, it could be possible to say that the differential gene expression of heterogeneous mRNA populations produced in CT, IT and HT of albedo and felavedo tissue, are quite similar except for those distinguishable proteins, which appeared in each types of albedo and flavedo tissue as previously described. These distinguishable proteins (displayed in this work) are representing the response of regulation process and inducing biochemical pathways of cellular system during the transition from healthy to creased phase but other similar (undistinguishable) proteins were unstimulated protein bands overwhelmingly due to any other physiological process than the effective creasing factor with no new induced biochemical pathway. These results are consistent with what had been mentioned by Alonso *et al*., 1992, Basharat *et al*., 2014, Gaynor & Cowan, 1996, Guirgis, 2000, Jacobsen *et al*., 1986, Krebs, 2012, Li *et al*., 2009, Lliso *et al*., 2007, Vierling, 1991 and Ziegler, 1990.

Determination of PG-ase activity

Figure 2 demonstrated clearly that greatest value of polygalacturonase activity (PG-ase) was resulted in albedo tissues taken from creased part of creased fruit and obviously it was higher than the values of (PG-ase) either of albedo from non-creased fruits or albedo from non creased part of creased fruit in both the investigated seasons.

In this respect, Monselise *et al*. (1976) revealed that pectinmethylestrase activity was highly promoted in albedo tissues of creased fruits which usually associated with a significant increase in water soluble pectin. Also, Basharat *et al*. (2014) on Washington navel and navelina orange fruits demonstrated that the activities of PE, exo and endo PG and Endo-1, 4-ß -D-glucanase were higher in the albedo tissue of creased fruit at different fruit maturation and ripening stages, and the elevated activities of pectinesterase, exo- polygalacturonase, endo- polygalacturonase, and Endo-1, 4-ß -D-glucanase in albedo and flavedo of creased fruit than healthy ones at harvest appear to be associated with the enhanced loss of pectin and starch in the cell walls of albedo, consequently reducing hardness, stiffness and tensile force of the rind possibly leading to cell wall loosening and fruit cracks formation.

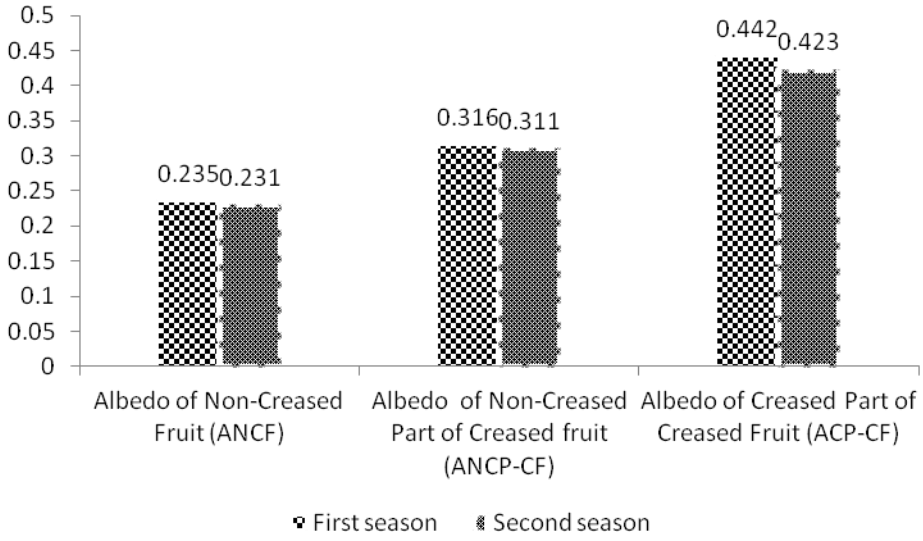

Fig. 2. PG-ase enzyme activity in ANCF, ANCP-CF and ACP-CF of Washington navel orange fruits in the two experimental seasons.

Creasing incidence percentage

Figure 3 showed the correlation between fruit geographical directions *i.e.* north or south and the creasing percentage (CP) in the three measuring dates, the creasing development from Jan. to March, and the trend line of each experimental season. The obtained results cleared that there is a positive correlation between the fruits situated in the northern half of tree periphery and the CP where the obtained values were higher. The data trend line also showed that the fruit CP has increased from the first measuring date (mid. Jan.) to the higher level in the last date (first of March).

It is noticed that the leaf density in the northern part of the tree is lower than the southern part. The same notice was true for the branches which hold creased fruits (observational data). This leaf density- reduction reduces leaves assimilate and/or specific elements transitions such as (Ca, K and S) to fruits causing an increase in fruit CP. Some of these elements might be also (directly or indirectly) responsible for signaling system in the plant cells (Fatma, 2009 and Yi Wang & Wei-Hua Wu, 2013) and also it is involved in the production of specific enzymes i.e. PE, exo and endo PG and Endo-1, 4-ß -D-glucanase related with the creasing symptoms development.

Regarding the fruit age, it is quite evident that the fruit CP is directly proportional to the fruit age. This might be due to the fruit senescence where the creasing disorders are subsequently decoded by cytoplasmic sensors, which regulate the downstream transcriptional and posttranslational responses (Yi Wang and Wei-Hua Wu, 2013). Consequently, the peel tissue cells are deteriorated. Moreover, it is true that as the CP increases it promotes the fruits to enter the senescence phase faster.

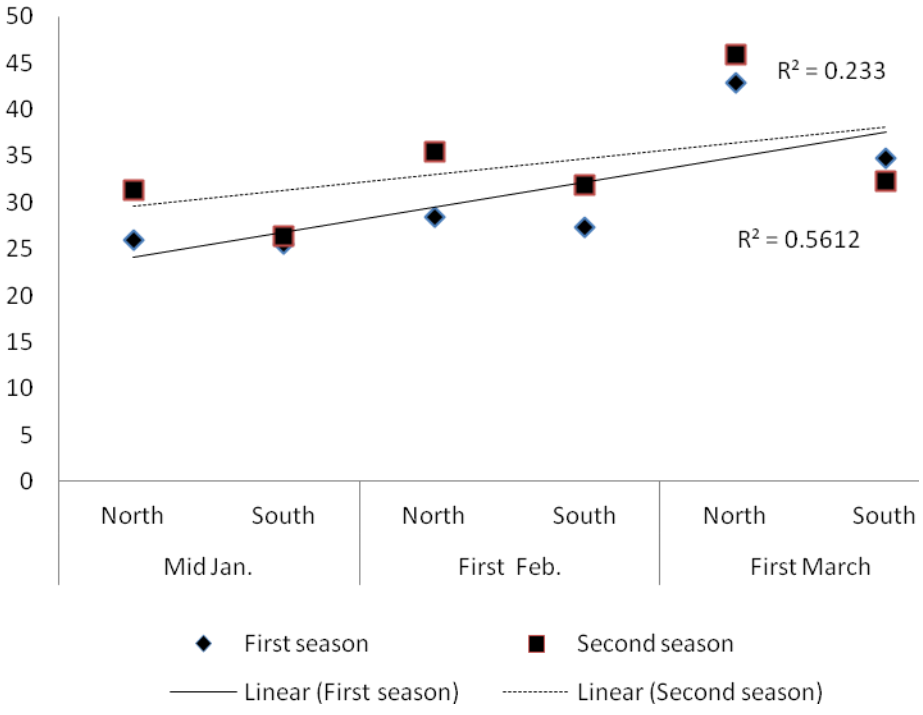

Fig. 3. Periodical changes in creasing percentage (CP) of Washington navel orange fruits as affected by geographical direction .

The obtained results are in harmony with El-Mahmoudi and El-Zorkani (1971) and Smit (1987) who mentioned that fruits of the shaded tree side (the northern part) were more susceptible to creasing than the fruits from the southern side due to either the higher relative humidity (RH) or the shading.

Moreover, Zou and Xu (1995) reported that, in cv. Hongjiang, differences were found between fruits exposed to the sun on the crown periphery and those heavily shaded in the depth of the crown, and between the half of the fruit exposed to the sun and the other half of the same fruit. These differences might be primarily responsible for differences in cracking rates.

Physical properties

Table 2 shows the physical quality properties of the creased and non-creased fruits in both experimental seasons.

TABLE 2. Creased and non-creased fruit physical proprieties of Washington navel orange.

Fruit state	Fruit Weight (gm)		Fruit volume (cm^3)		Fruit Polar diameter (cm)		Fruit equatorial diameter (cm)		Fruit shape index		Peel thickness (cm)	
	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2010	2011
Sound	243.42	238.33	266.33	250.67	7.94	7.73	7.66	7.68	1.04	1.01	0.47	0.49
Creased	204.27	196.63	214.33	212.77	7.19	7.18	7.17	7.13	1.00	1.00	0.26	0.27
LSD 0.05	17.17	9.59	19.27	9.67	0.235	0.24	0.195	0.09	0.014	N.S.	0.04	0.039

The obtained data showed significant increments in fruit weight (g), volume (cm^3), fruit dimensions *i.e.* fruit polar and diameter in the non-creased fruits. Concerning the fruit shape index there were no significant difference between the creased and non-creased fruits in the second season. However, the fruit peel thickness of the non-creased fruits was significantly thicker than the creased one in both seasons. This is true since the pectin layer were broke-down in the creased fruit due to the catalectic enzyme activities. Yet, the creased fruits were hanged on branches located with less leaves and light intensities. These results are similar to once obtained by Sharaf *et al*. (2005) and Fatma, 2003 and 2009 who pointed out that, creased fruits of Hamlin orange, Washington navel and Tanarif orange cultivars had significantly thinner peel as compared to non-creased ones.

An explanation for the increase in fruit weight and volume in the non-creased fruits compared to creased ones could be due to the increments in water

and elements translocations since the non-creased fruits were attached on branches with more leaves density than the others, or it could be related to the best aeration and light intensity exposure.

Fruit chemical properties

Results in Table 3 summarize the differences between creased and non-creased navel orange fruits in the total soluble solids TSS (%), acidity (%), TSS/acid ratio and vitamin C content.

TABLE 3. Creased and non-creased fruit chemical proprieties of Washington navel orange.

Fruit state	TSS (%)		Acidity (%)		TSS/acid ratio		Vit. C (mg/100g)	
	2010	2011	2010	2011	2010	2011	2010	2011
Non-creased	12.17	12.33	0.99	1.07	12.29	11.52	51.67	57.5
Creased	12.77	13.83	0.91	0.89	14.03	15.54	50.84	59.79
LSD 0.05	0.199	0.561	0.045	0.06	0.66	0.98	2.95	3.51

It is noticed that fruit TSS increased fruits was higher than in non-creased ones, while the fruit acidity has the opposite trend in both seasons.

On the whole, it is well-known that any type of stress the fruits are exposing to, increases the fruit juice TSS. This might be due to the increments in this enzyme (PG-ase, PE, exo and endo polygalacturonase (exo-PG, endo-PG), and endo-1, 4-ß -D-glucanase (EGase)) which solute the insoluble solids into soluble increasing the cell osmotic pressure as a sort of cell defense against unfavorable factors Basharat *et al*. (2014). These results support those reported by Salama (1979) on some fruit varieties who reported that, the cracked fruit juice contained higher soluble solids, and lower total acidity than non-cracked fruits. Moreover, Fatma (2003 and 2009) demonstrated that there is a close relationship between physical and chemical characteristics of Hamlin orange fruits and the creasing incidence.

Conclusion

The results of the present study reported herein has approached and shed light on better understanding of the creasing disorder. Creasing is considered as the response of plant cellular and genetic systems to certain stress factors. These stress factors resulted in the production of specific creasing proteins (25, 26, 31, 32 and 35 kDa). The study, also, revealed that creasing is passing by an

important intermediate stage (characterized by the existing of 69 kDa protein) which can be used as an early warning system indicating that the cell is in the transition phase towards creasing where the fruits morphologically seem not showing creasing disorder. In addition, we were able to identify other specific proteins (51-37) supposed to be responsible to prevent the incidence of fruit creasing. Once these proteins started to disappear, the cell started to convert into the intermediate stage.

The results also showed that creasing depends on some external factors *i.e.* geographical location (north side more than south side), shade, aeration, humidity, light intensity, specific agricultural practices and mineral contents of some nutrients such as calcium, potassium and sulfur causing other internal actions *i.e.* the existing of the newly identifying proteins and specific enzymes.

Even though, the fruit chemical properties are better in the creased fruits than in the non-creased ones yet it has no economical value. The creased fruits represent considerable economic loss since they are a non marketable and therefore, remain in the local markets or discarded.

Based on our results, further investigations are also needed to clarify the (51-37) proteins which may be related to the acquisition of creasing disorder.

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(Received 21/8/2014;
accepted 24/9/2014)

دور المحتوى البروتينى و النشاط الإنزيمى فى ظاهرة التبحير فى ثمار البرتقال أبو سرة واشنجطن

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أجريت دراسة بحثية لمدة موسمين متتاليين لرصد وتقييم البروتين و النشاط الإنزيمى في ثمار البرتقال بسرة واشنجطن السليمه و كذلك المصابه بالتبحير في الدلتا، مصر . حيث تم قياس الخواص الفسيولوجيه و الكيميائيه للثمار و كذلك النسبه المئويه للتبحير فى الثمار فى ثلاثة مواعيد متتاليه (منتصف يناير ، أول فبراير ، أول مارس) و فى إثنين من الاتجاهات الجغرافية للشجرة (الشمال و الجنوب) و أثر الإتجاه الجغرافى على نسبة التبحير فكانت أعلى فى ثمار النصف الشمالى من الشجرة عنه فى الجنوبى .

وفي الوقت نفسه ، فإن أنماط البروتين الكلى فى كل من الألبيدو والفلافيدو أوضحت أن هناك ارتباط بدرجات مختلفه بين كل من نوع البروتين و حالة الثمرة سليمه أو مبحرة وعلاوة على ذلك لوحظ ارتفاع معدل نشاط إنزيم بولى جالاكتويورينيز (PG-ase) فى ألبيدو الثمار المبحره مقارنه بألبيدو الثمارغير المصابه بالتبحير.