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Mechanisms of Chilling Injury of 'Marsh' Grapefruit (*Citrus paradisi* Macf.)

Hameed Jasim Al Juburi
Plant Production Department
Faculty of Agricultural Sciences
UAE University, Al-Ain

Abstract: Scanning electron microscope photomicrogaphs of "Marsh" grapefruit peels showed that severe chilling injury was a depressed area of collapsed cell, just beneath the epidermis layer. As the severity of chilling injury increased, non-collapsed cells gradually increased in size. Cell walls were irregularly shaped; thin in some areas and thick in others. Oil glands in depressed areas were not ruptured during cold storage. Hence release of toxic materials through rupturing of oil glands is not a factor in chilling injury. Compared to noninjured tissue, injured tissue from the same fruits had significantly lower water and osmotic potentials, and low, near zero, turgor pressures. There was a significant negative correlation ($r = -0.5$; $p < 0.05$) between the water potentials of the peels and the percentage of "Marsh" grapefruit with severe chilling injury during storage at 2.8°C. Ion leakage tended to increase during cold storage, and when the fruit were transferred to room temperature. Internal conductivities declined sharply during cold storage and then increased very strongly during periods at room temprtature.

Additional key words: Grapefruit, *Citrus paradisi,* chilling injury, water components, cell size, oil gland, scanning microscopy, ion leakage, and electrical conductivity.

INDRODUCTION

Ultrastructural changes have been associated with chilling injurey of horticultural crops, when grape fruit and tomato (fruit and seedlings) were exposed to low temperatures organelle structures were extensively

modified, chloroplast conversion to chromoplast was inhibited, mitochondria were swollen, cytoplasms became more vacuolized, cell volume changes, and cell walls showed irregular shape (Ilker et al., 1979).

Microscopic studies of chilling injury of orchid leaves cv. pink chiffon) showed that the initial response to chilling injury occurred in mesophyll cells between vascular bundles. However, tissues with a severe chilling injury were characterized by extensive mesophyll collapse, which often caused a slight depression of the epidermal cells (McConnell and Shaheen 1978).

Chilling injury enhanced leakage of various ions from many susceptible tissues (Chan et al., 1985; Chen and Paul, 1986; Furmanski and Buescher, 1979; Greencia and Bramilage, 1971; Murata and Tatsumi, 1979; Pantastico, 1968; Wright, 1974 and Wright and Simon, 1973). However electrolytic conductivities of leachats from limes and grapefruit (chilling senstive fruit) were three times higher than those of leachate from orange fruit tissues, which are relatively resistant to chilling injury (Pantastico, 1968). Studies of chilling effects on electrolyte leakage and internal conductivities of peach fruit (Furmanski and Buescher, 1974) and pepper and egg plant (Murata and Tatsumi, 1979) showed that the chilled fruit did not exhibit any increase in electrolyte leakage.

High humidity alleviated chilling injury and low humidity aggravated the symptoms in limes and banana (Pantastico, 1968), and in most vegetables and fruits (Grierson and Wardoski, 1978 and Vakis et al, 1970). Low humidity enhanced chilling injury in many commodities and this could be due to partial dessication of tissues.

The objective of this study was to investigate periodic warming, calcium chloride ($CaCl_2$), abscisic acid (A B A) and 2,4 - dichlorophenoxyacetic acid (2,4-D) for their ability to reduce chilling injury, as well as to gain some understanding of the mechanisms of chilling injury by studying ion leakage, internal conductivity, water potential components, and scaning electron micrograph of injured tissue.

MATERIALS AND METHODS

The university of Arizona Reserch farm at Tempe was furnished of 600 mature "Marsh" grapefruit (late october 1981) which were shipped

in carton to Tucson. The fruit were washed and sterilized with 1:4 v/v commercial bleach for 3 minutes, then rinsed with tap water and left to dry over night. The fruit were randomly divided into twelve treatment, each treatment consisting of 40 grapefruit. Two hundred fruit were dipped for 15 second at room temperature in a solution containing 0.2% Triton, × 100, then they were left to dry for 6 hours at room temperature. One drop of benomyl 0.5g/L was added to each fruit at the point of detachment. Each treatment was placed in acarton and stored at 2.8°C. One treatment was left in the storage room with out any warm period as a control. Fruit. for other treatments were stored at 2.8°C with one day at room temperature for every, 4, 8, 12, and 16 days in cold storage.

The other two hundred eighty grapefruit were immersed in one of the following solution:

0.14M $Cacl_2$, 10^{-4} M ABA, 4×10^{-4} M 2,4-D, or in combinations of ABA and 2,4-D, 2,4-D and $Cacl_2$, or ABA and $Cacl_2$ containing 0.2% Triton x-100 for 3 miuntes. The fruit were then dried and treated with benomyl as above, and stored in cartons at 2.8C° for further study.

Periodically, one grapefruit was removed from each treatment (first treatment was stored at 2.8C° without warming periods, and others were stored at 2.8C° (with one day at room temperature for every 4, 8, 12, 16 days) to be testing for ion leakage. Each fruit was peeled with a sharp razor blade and 15 discs (2 mm thickness and 12 mm diameter) were removed from each grapefruit peel under distilled water with a sharp cork borer. The discs were incubated in a 50 ml Erlenmeyer flask containing 30 ml of 0.4 M Mannitol for 3 hours at 30C° in a shaking water bath at a shaking speed of 40 oscillations/minute. Ion or eletrolyte leakage into the mannitol solution was determined with a conductivitty bridge sensitive btween 0 and 100 umhose. After the 3 thours at 30°C the slices were killed by boiling the solution containing the discs for 15 minutes in an oven and then cooling to 30C°. The conductivity of the solution was then redetermined. The latter conductivity reading represented the total ion leakage. The ion leakage of living tissue was then calculated as a percentage of the total ion leakage (Greencia and Bramlage, 1971).

Internal conductivity changes of "Marsh" grapefruit peels were determined periodically. A conductivity bridge was used with a probe consisting of two needles set 5 mm apart in a rubber stopper so that they could be inserted into the grapefruit peel to a depth of 3 mm. Internal conducitvity was measured at four positions around the stem end, the stylar end the equater, and the four values from each were averaged. Reading were in umhos/sec, calculated by the seconds required to reach 45 mhos.

Water potentials are measured with a Wiscor thermocouple psychrometer (Model M.J.55) by removing slices 1 mm thick and 5 mm in diameter from the peel with a sharp cork borer, and then removing iols by wiping with kimwipe tissues. Groups of four slices were then placed directly in the thermocouples with flavedo up and albedo down. Psychrometer readings (check, 10^{-4}M ABA and 4×10^{-4} M 2,4-D, and 0.4 $cacl_2$ separately or in combination) were made weekly on randomly selected fruit.

Similarly obtained peel slices were sealed in small aluminum foil vials and frozen in liquid N_2 and stored in the freezer overnight. Water potentials for these killed tissues are equivalent to the osmotic potential of the cells.

Turgor pressure were calculated as the difference in water and osmotic potentials. "Marsh" grapefruit were peeled with a sharp razor blade and sections of 5 mm^2 and 1 mm thick were dissected from injured and non-injured areas of the equator and fixed at room temperature for 2 hours with 2.5% glutaraldehyde and 0.1 M potassium phosphate buffer (PH 7.4) for 1 hour. The tissues were rinsed in the buffer to remove the glutaraldehyde, and postfixed for 1 hour with 4×10^{-4} M osmium tetroxide (OSO_4) in 0.1 potassium phosphate (pH 7.4). The tissues were dehydrated with an acetone series.

Specimens were placed in the critical point dryer apparaturs in acetone, and carbon dioxide was used as the transitional liquid (Dawes, 1979). The dried specimens were stored over a desiccant until they were mounted on stubs by using colliodal silver (Pelco-Ted Pella Co.) and the coated with carbon and then with an alloy of palladium and gold by

using a sputter coater (Modle SEM Coating Unit E 5100, Polaron Instrument, Inc.). Then the specimen was placed in the scanning electron microscope (Model ISIDS 130). Samples were observed at various magnifications and zero tilt at 20 Kev, while operating in secondary electron mode.

RESULTS AND DISCUSSION

Electrolyte leakage tended to decline during storage at 2.8C°, reaching the lowest levels after 32 days storage (Table 1). Transferring the fruit to room temperature for a short holding period resulted in significant increases in electrolyte leakage after a 2 day lag. After reaching a maximun of 52.9%, after 8 days at room temperature electrolyte leakage declined to 43.3%.

Internal conductivities of fruit peels at the stem end, stylar ends and the equator tended to decline sharply during storage reaching manimum values after 2 days at room temperature following 51 days at 2.8C° at all positions. The stem ends had the highest values, and the stylar ends the lowest (Fig). 1).

Internal conductivities for the grapefrut declined during 45 days at 2.8C° for all treatments (Table 2). Transferring the fruit to room temperature after 51 days at 2.8C° did not result in a significant increase in internal conductivity during the first 2 days. However, during 10 days at room temperature following 51 days at 2.8C°, internal conductivities increaced for all treatments, but less for fruit warmed every 16 days than for control fruit (Table 2). Fruit warmed every 12 days had considerably higher internal conductivities.

Ion leakage remained somewhat constant during 40 days in darkness at 2.8C° then increased. Transferring the fruit to room temperature after 51 days cold storage further enhanced ion leakage from "Marsh" grapefruit peel discs into 0.4 M mannitol solution, for 8 days, then ion leakage decline during storage in darkness at 2.8°C for 51 days. Moving the fruit to room temperature, resulted in a rapid increases in internal conductivities of fruit after a 2 day lag. Internal conductivities reached their highest valuse 10 days after transfer from cold storage (Fig. 2).

Table 1 Changes in ion leakage of 'marsh' grapefruit flavedo during cold storage and subsequent holding at room temperature.

Time between 24 hrs Warm preiods (Days)	Ton Leakage[z] (Z of Maximum)							
	Total Days in Cold Strcrage					Days at Koom Temperature Following 51 Days at Cold Storage		
	21	24	32	38	45	2	8	10
Continuous Cold	33.8 ± 4.1 bcy	32.9 ± 2.6 bc	24.4 ± 5.9 a	32.6 ± 1.8 bc	35.5 ± 2.8 c	31.5 ± 5.6 b	52.9 ± 3.2 a	43.3 ± 3.7 d
16	31.03 ± 7.2 b	30.3 ± 2.6 b	32.5 ± 2.0 b	24.2 ± 1.6 a	40.1 ± 3.0 c	38.5 ± 4.2 c	54.3 ± 0.8 d	52.9 ± 3.9 d
12	38.3 ± 1.2 c	32.6 ± 3.2 b	31.9 ± 3.8 b	28.4 ± 4.1 a	29.6 ± 3.5 c	45.4 ± 1.5 d	49.7 ± 1.8 e	43.1 ± 3.5 d
8	34.5 ± 2.0 b	32.8 ± 1.5 b	32.0 4.7 b	28.0 ± 5.2 a	42.7 ± 3.0 c	46.6 ± 3.1 d	52.3 ± 3.5 e	40.7 ± 4.3 c
4	39.5 ± 3.4 c	28.6 ± 2.3 a	40.0 ± 0.6 c	32.5 ± 1.8 b	52.4 ± 3.3 c	45.4 ± 3.1 d	56.5 ± 2.4 f	48.6 ± 1.1 d

[z] Each value represents the average of 4 readings, one each from 4 fruit.

[y] Means within a row followed by the same letter are not significantly diffrtrny at the 5% level according to Duncan's Multiple-Range Test.

LSD = 3.2

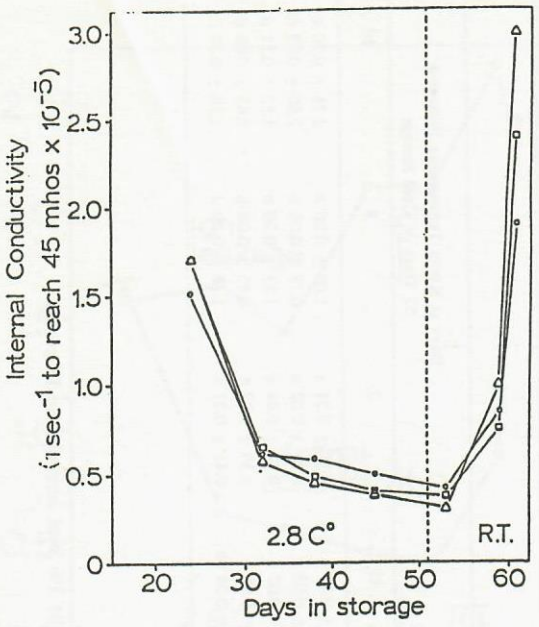

Figure 1 Internal conductivities of "Marsh" grapefruit peels during 51 days at 2.8C° followed by transfer to room temperature (R.T.). Internal conductivities were determined at room temperature at the stem-end (△ - △), equaror (0 - 0) and staylar end (□ - □). Each value represents an average of 80 measurements (4 each from 20 fruit).

These results of ion leakage are in agreement with those of Chan and Buescher (1979) and Murata and Tatsumi (1979), who found that ion leakage from papara fruit, tomato fruit, peach fruit, egg plant and bell pepper fruit remained fairly constant during 5 weeks or 3 weeks at 1 C° or 2 C° respectively, and increased when peach fruits were moved to 21 C°. Results of internal conductivities also are consistent with those of Furmanski and Buescher (1979), who found that internal conductivity of peach fruit declined during storage at 1 C°. Moving the fruit to 21 C° greatly increased internal conductivities.

Table 2 Changes in internal conductivity of 'marsh' grapefruit flavedo during cold storage and subsequent holding at room temperature.

Time between 25 hrs Warm preiods (Days)	Internal Conductlvity[z] (umohs)						
	Total Days in Cold Strcrage				Days at Koom Temperature Following 51 Days at Cold Storage		
	24	32	38	45	2	8	10
Continuous Cold	1.40 ± 0.10 by	0.33 ± 0.04 a	0.35 ± 0.03 a	0.20 ± 0.03 a	0.38 ± 0.01 a	1.08 ± 0.06 b	2.75 ± 0.20 c
16	1.23 ± 0.09 c	0.43 ± 0.93 a	0.38 ± 0.02 a	0.28 ± 0.02 a	0.28 ± 0.02 a	0.78 ± 0.02 b	2.00 ± 0.07 d
12	2.23 ± 0.20 c	0.55 ± 0.03 a	0.40 ± 0.04 a	0.30 ± 0.02 a	0.33 ± 0.04 a	1.23 ± 0.20 b	4.53 ± 0.51 d
8	1.45 ± 0.02 c	0.50 ± 0.04 ab	0.48 ± 0.06 ab	0.63 ± 0.04 b	0.30 ± 0.03 a	0.65 ± 0.08 b	2.63 ± 0.08 d
4	2.08 ± 0.23 e	1.03 ± 0.14 ed	0.75 ± 0.16 bc	0.45 ± 0.04 ab	0.43 ± 0.01 a	1.18 ± 0.20 d	2.78 ± 0.30 f

[z] Each value represents an average of 16 readings, one each from 4 fruit.

[y] Means within a row followed by the same letter not significantly diffrtrny at the 5% level according to Duncan's Multiple-Range Test.

LSD = 0.3.

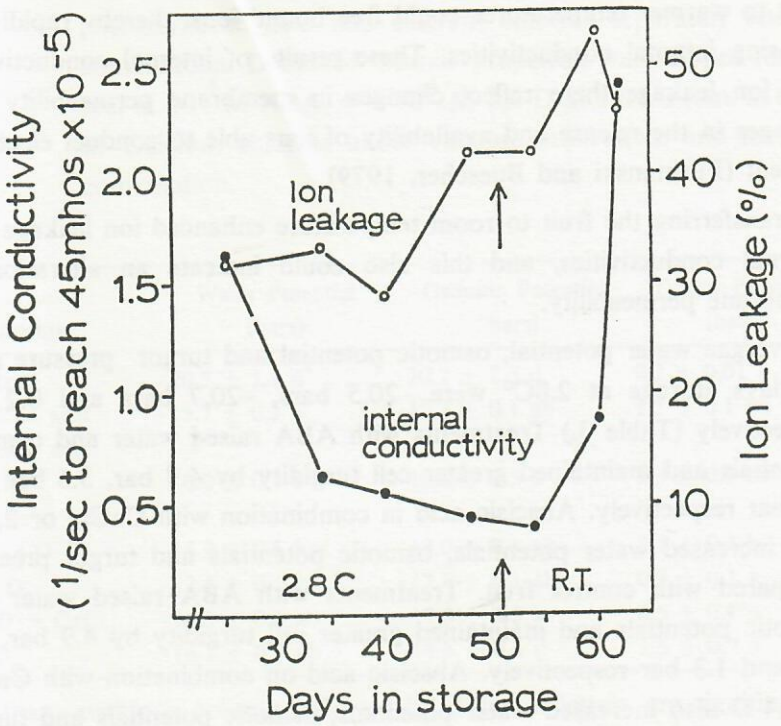

Figure 2 Electrical conductivity and ion leakage of "Marsh" grapefruit peels during storage. Fruit were stored for 51 days at 2.8C° and then transferred to room temperature (R.T.). Internal conductivity values are mean of 240 readings (4 each from 60 fruit). Ion leakage values are means of 20 readings.

Storage at low temperatures has enhanced ion leakage from many chill sensitive tissues (Cahn et al, 1985; Chen and paull, 1986; Greencia and Bramlage, 1971; Lyons, 1973 and Wright and Simon, 1973). Enhancement of ion leakage has been attributed to the promotion of membrane permeability (Farmanski and Buescher, 1979 and Lyons, 1973).

However, electrical conductivity data suggest that free ions could be increasingly bound with increasing time at low temperature, moving the fruit to warmer temperatures could free bound ions, thereby rapidly increasing internal conductivities. These results of internal conductivities and ion leakage there reflect changes in membrane permeability and changes in the release and availability of ions able to conduct electrical current (Furmanski and Buescher, 1979).

Transferring the fruit to room temperature enhanced ion leakage and internal conductivities, and this also could indicate an alteration in membrane permeability.

Avergae water potential, osmotic potential and turgor pressure after 42 days storage at 2.8C° were - 20.5 bars, - 20.7 bars and 0.2 bar respectively (Table 3.) Treatments with ABA raised water and osmotic potentials and maintained greater cell turgidity by 4.9 bar, 3.6 bar and 1.3 bar respectively. Abscisic acid in combination with $CaCl_2$ or 2,4-D also increased water potentials, osmotic potentials and turgor pressure compared with control fruit. Treatments with ABA raised water and osmotic potentials and maintained greater cell turgidity by 4.9 bar, 3.6 bar and 1.3 bar respectively. Abscisic acid on combination with $CaCl_2$ or 2,4-D also increased water potentials, osmotic potentials and turgor pressure compared with control fruit. Treatments with ABA in combination with $CaCl_2$, significantly raised water potentials, osmotic potentials and turgor pressure by 2.6 bar, 1.5 bar, and 1.1 bar respectively (Table 3), and ABA in combination with 2,4-D also increased water, osmotic potentials and turgor pressure to - 16.8 bar, - 17.95 bar and 1.1 bar, respectively, but 2,4-D alone only increased water and osmotic potentials and did not change turgor pressures. Similar results were obtained when 2,4-D was used in combination with $CaCl_2$ to fruit increased water and osmotic potential, and turgor pressure significantly by 3.8 bar, 2.4 bar, and 1.4 bar.

Comparing water potentials with the percentage of 'Marsh' grapefruit with severe chilling injury during 4 weeks storage at 2.8°C, showed a significant negative correlation ($r = 0.5$; $p < 0.05$). The means, high water potentials are associated with a low degree of chilling injury. After 14 days at 2.8 C° water potentials were - 18 bar, and the percentage of

Table 3. Water potential components of 'Marsh' grapefruit after 42 days cold storage in respons to abscisic acid, 2.4-Dichlorophenoxyacetic acid and calcium chloride separately and in combination. Detached 'Marsh' grapefruit were dipped for 30 sec. in 10^{-4} M ABA, 4×10^{4} M 2,4-D and for 3 min. in 0.14 M Calcium chloride under vacuum infiltration. and then in combination.

Treatments	Water Potential (bars)	Osmotic Potential bars)	Turgor Pressure (bars)
Control	-20.5 ± 1.3 d[z]	-20.7 ± 1.3 d	0.2 ± 0.01 a
Abscisic acid	-15.7 ± 0.3 a	-17.2 ± 0.1 ab	1.5 ± 0.1 bc
Abscisic acid $CaCl_2$	-18.0 ± 0.1 c	-19.3 ± 0.5 c	1.3 ± 0.7 bc
Absisic + acid + 2, 4-D	-16.9 ± 0.6 bc	-18.0 ± 0.1 ab	1.1 ± 0.1 b
2, 4-D	-17.4 ± 0.1 c	-17.7 ± 0.1 ab	0.3 ± 0.1 a
2,4-D + $CaCl_2$	-16.5 ± 0.4 ab	-17.0 ± 0.3 a	0.5 ± 0.1 a
$CaCl_2$	-16.7 ± 0.3 ab	-18.3 ± 0.4 c	1.6 ± 0.7 c

[z] Means within a column followed by different letters are significantly different at 5% level according to the Duncan's Multiple Range Test. Each reading represents the mean of 20 measurements.

fruit with severe chilling injury was zero. After 42 days at 2.8 C water potentials of the fruit had decreased to-28 bar and the percentage of fruit with severe chiling injury had increased to 50% (Fig. 3).

A comparsion between water potential components of injured and sound areas of the same fruits, showed that injured spots had low water potentials, osmotic potentials and turgor pressures near zero. Healthy areas of fruit had higher water potentials, Osmotic potentials and turgor pressure compared with tissue from unhealthy spots (Table 4). Significant differences in water potential components of injured and non-injured peel tissues suggests that water loss is at least one factor in the on-set of chilling injury in 'Marsh' grapefruit. Therefore, these results

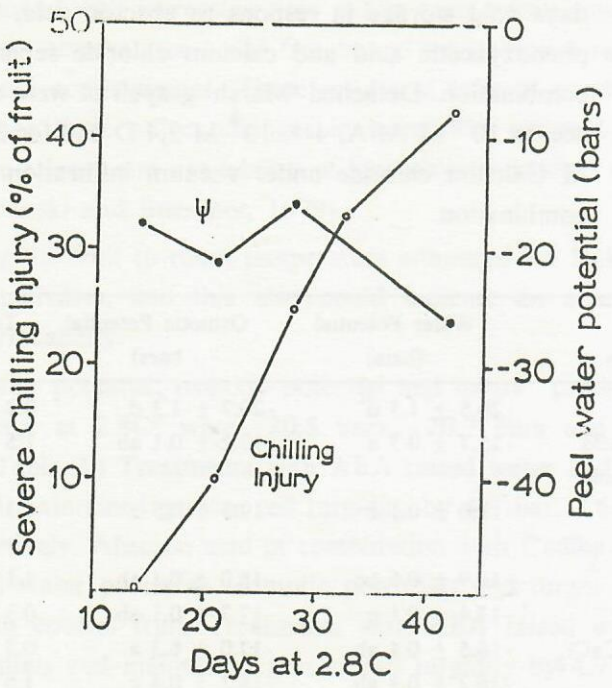

Fig. 3. Water potential (ψ) and development of severe chilling injury to 'Marsh' grapefruit peels during cold storage.

could support the hypothesis that a high relative humidity in storage room reduce chilling injury by reducing water loss via transpiration.

Membrane permeability could increase during the chilling period (Greencia and Bramiage 1971 and King and Ludford, 1983) allowing a loss of water, which evaporate off, and solutes, which remain in cell wall, could cause chilling injury. Therefore high humidity or agents that reduce water loss may simply suppress the chilling injury.

Table 4. Water potential components of injured and non-injured tissue from "Marsh" grapefruit.

Source of Tissue	Water Potential (bars)	Osmotic Potential (bars)	Turgor Pressure (bars)
Non-injured	-17.5 ± 0.3 a	-18.8 ± 0.3 a	1.3 ± 0.3 a
Injured	-27.3 ± 1.0 b	-27.6 ± 1.0 b	0.3 ± .1 b

[z] Means within a column followed by different letters are significantly different at 5% level.

These results are consistent with those of previous studies for cucumber seedlings (Rikin et al., 1976) and for tobacco plants (Boussiba et al., 1975). Walton et al. (1977) found that the transpiration and water loss decreased progressively with increase in ABA content whether induced by water stress or by an exogenous application. So the reduction in water loss could be the reason for reducing chilling injury by ABA application (Boussiba et al., 1975 and Rikin et al., 1976). Calcium chloride could act as osmotic active solute or by reducing membrane permeabilities and hence leakage (Bangerth et al. 1972). So plant growth regulators and $CaCl_2$ could act in reducing chilling injury by improving water balance of commodities in storage room.

Scanning electron microscope photomicrographs of grapefruit peels, including oil glands and injured and non-injured areas revealed that the commenest symptom of chilling injury was sunken areas in peels (Fig. 4). The depression could be as a consequence of swollen cells beneath the epidermis cells, which could press on the upper cells causing them to collapse and resulting in the depressed areas. As the peel began to sink down and the epidermis cells started to collapse there was a slight increased in cell size below the epidermis cell (Fig. 5A and B.) As the sunken area increased in depth, the number of crushed epidermal cells increased in size by threefold (Table 5 and Fig. 5C and D). The cells became irregularly shaped when chilling injury was severe. Cell walls were thick in some areas and thin in others (Fig. 5C) and cutting of

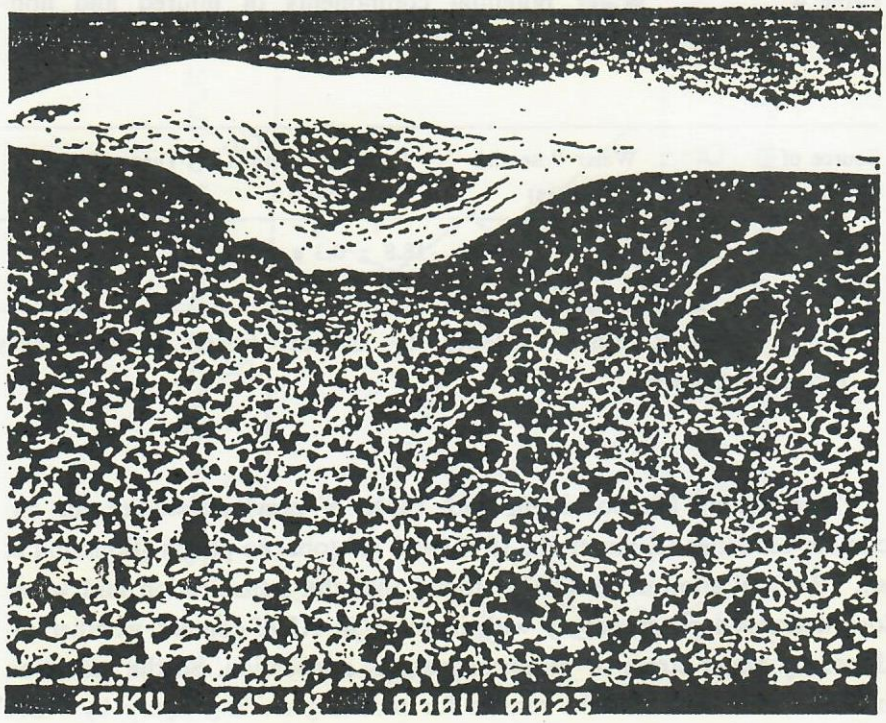

Fig. 4. Scanning electron micrograph of pitted area from 'Marsh' grapefruit peel with severe chilling injury.

sections resulted in more ragged cell edges. It is important to note that oil glands were not ruptured during cold storage. Photomicrogaphs showed that oil glands were not broken down even in fruit with severe chilling injurt (Fig. 6), so we can reject the hypothesis that 'Marsh' grapefruit oil glands ruptured during cold storage, and released their oils causing cell death and the onset of chilling injury symptoms.

These results are similar to those of Ilker et al (1979), who found from electron microgaphs of tomato cotyledon cells exposed to chilling temperature, that the cell wall showed marked irregularities and the cell wall appeared thinner in some areas and expanded in others, with increase material in the middle lamella, and those of McConnell and Sheehan

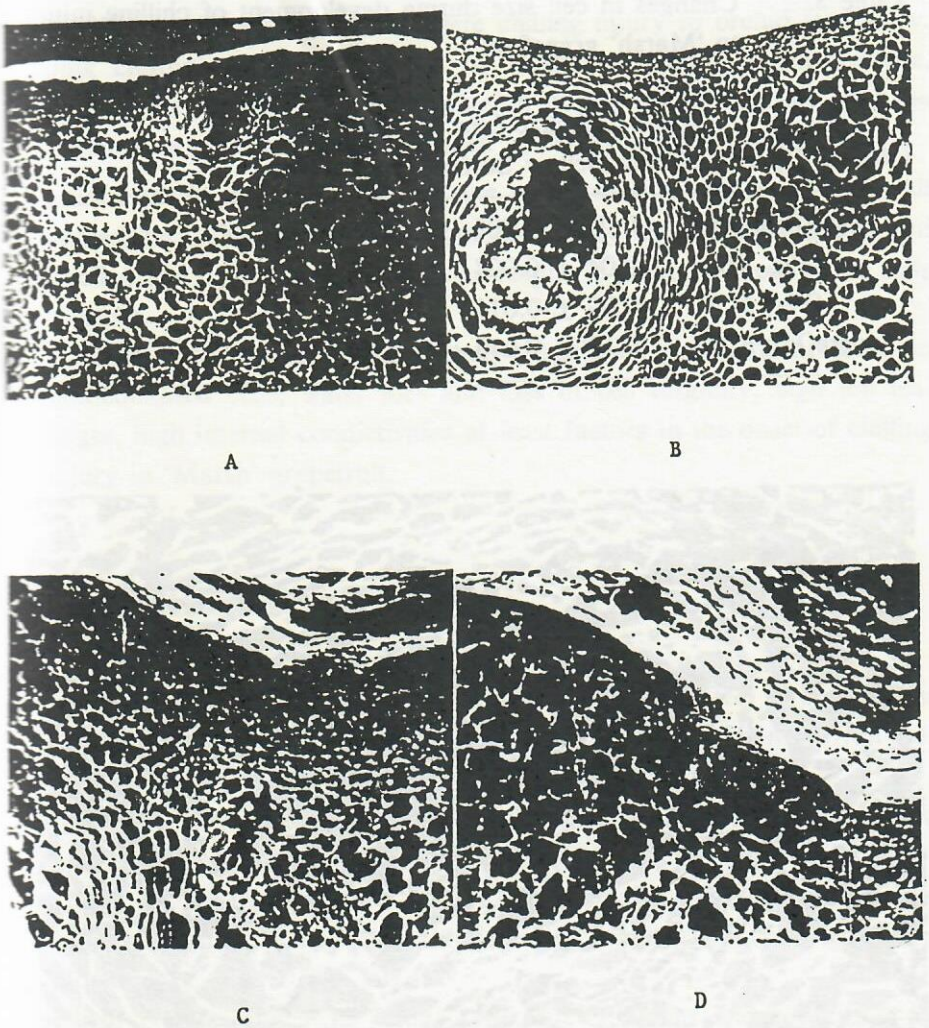

Fig. 5. Scanning electron micrograph of the developing depression in 'Marsh' grapefruit peel with chilling injury.
Early stage of injury (A,42X) and progressively deeper depression (B, 42X; C and D, 31X) of peel due to chilling injury.

Table 5. Changes in cell size during development of chilling injury to 'Marsh' grapefruit.

Chilling Injury	Mean Cell Size (μm^3) 30 mm Bellow Surface	Cells/cm^2 of Flavedo
Slight(Fig.5A	0.03 ± 0.006	38025 cell/cm^2
(Fig. 5B)	0.04 ± 0.004	33800
(Fig. 5C)	0.09 ± 0.015	16900
Severe(Fig.5D)	0.12 ± 0.013	12675

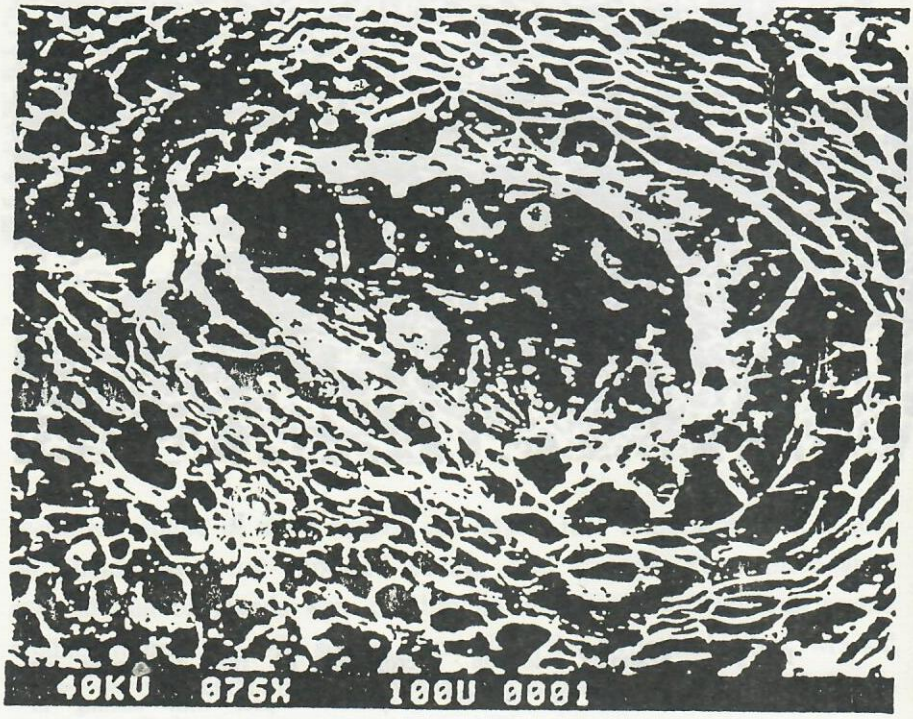

Fig. 6. Scanning electron micrograph of oil gland from peel of 'Marsh' grapefruit with severe chilling injury.

(1978) who found that more severe chilling injury to orchid leaves (cv. pink chiffon) was characterized by extensive mesophyll cell collapse, which often caused a slight depression of the epidermal cells, and the depressions were always surrounded by hypertrophied cells.

Increase in cell size could be due to increase in activity of cell wall degradtion. When cell walls become thin, they are easily expanded and swollen and cannot stand against atmospheric pressure, therefor the cells collapse.

These data suggest that cell walls irrgularity gradually increased in non-collapsed cells, water loss and loss of cell turgidity, high ion leakages, high internal condictivities at least factors in the onset of chilling injury in 'Marsh' grapefruit.

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