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Water Loss: A Nondestructive Indicator of Enhanced Cell Membrane Permeability of Chilling-injured *Citrus* Fruit

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Abstract. **Water loss was found to be a nondestructive indicator before visible symptoms of chilling injury (CI) in cold-stored grapefruit (*Citrus paradisi* Macf.) and lemon (*C. limon* L. Burm. f.). The water-loss rate increased significantly after removing the fruit from cold storage and holding at 20C. Scanning electron microscopy revealed large cracks around the stomata. Changes in electrical conductivity of the flavedo tissues, total electrolyte leakage, and K^+ or Ca^{2+} leakage were all inadequate predictors of CI, appearing only after CI was evident.**

Chilling injury (CI) of citrus fruit is characterized by localized discoloration of the peel followed by the collapse of the affected area, which results in depressions on the fruit surface. Lyons (1973) suggested that CI is the result of ultrastructural membrane changes that permit electrolyte leakage and ion imbalance in the cell.

The increase in total ion leakage, K^+ leakage specifically, was reported to be a sensitive indicator of CI in grapefruit callus tissue (Forney and Peterson, 1990) and in whole oranges, limes, and grapefruit (Pantastico et al., 1968).

Since ion leakage measurements are, by their nature, destructive and time consuming, an indicator of CI was sought that would be relatively rapid, reliable, and nondestructive. We therefore investigated whether water loss could serve as a reliable nondestructive indicator of CI in stored grapefruit and lemons. These measurements were then compared with those for total electrolyte leakage, K^+ and Ca^{2+} leakage, specifically.

Materials and Methods

'Marsh' grapefruit and 'Villa franca' lemons were harvested and, without any treatment, divided into three lots, each consisting of three cartons with 50 grapefruit or 100 lemons each. One lot was stored at 2C (a CI-inducing temperature), the second lot was stored at 13C (a nonchilling temperature), and the third lot was stored under an intermittent warming (IW) regime of 21 days at 2C followed by 7 days at 13C (Cohen et al., 1983) for 12 weeks. Relative humidity was maintained at ≈90% under all storage conditions.

Weight loss was determined by individually weighing 15 grapefruit or 25 lemons per treatment at harvest and at 4-week intervals during storage. Fruit were also weighed daily during the 4 days of holding at 20C after removal from storage.

Electrical conductivity (EC) was measured using stainless-steel electrodes, 5 mm apart, embedded in a rubber stopper and protruding 2 mm. EC was measured at five locations along the equator of three randomly chosen washed fruit in each treatment.

Membrane leakage was determined using fruit disks used for EC measurements. Five disks (10 mm in diameter, 3 mm thick) consisting of flavedo and albedo tissue were removed from each fruit and immersed in 15 ml deionized water at 20C with constant shaking. After 3 h of incubation, the electrolyte reading of the bathing solution was measured using a conductivity meter (model LF 535; Wissenschiftlich TechnischeWerkstatten GmbH, Germany). The K^+ and Ca^{2+} contents of the bathing solution were measured using ion-specific electrodes. The tissues and solutions were then held at –18C overnight followed by autoclaving (130C) for 15 min to permit complete leakage from the membranes. Percent leakage of total electrolytes, K^+, and Ca^{2+} was calculated as the ratio of the initial reading to the final reading.

CI was quantified by assessing the surface area and depth of pitting that developed on the peel. Light, moderate, and severe pitting were rated as 1, 2, and 3, respectively, and the CI index was calculated by the formula [(no. of fruit ranked 1×1) + (no. of fruit ranked 2×2) + (no. of fruit ranked 3×3)]/total number of fruit.

Scanning electron microscope (SEM) photographs from pitted and nonaffected disks of grapefruit peel were examined. Fruit were sampled after 3 months at 2C, with and without IW. Disks (4 mm) were critical-point dried after fixation in glutaraldehyde followed by osmium tetroxide and then serially dehydrated in ethanol. The samples were attached to the microscope stub, coated with gold, and observed at 10 to 15 kV, 0° tilt angle, and 15-mm working distance. External morphology of epidermal cell and wax structure was studied in a wide range of magnification: ×50 to ×15,000.

Results

Weight loss increased during storage, regardless of temperature. However, weight loss was greater in grapefruit (Fig. 1) and lemon (Fig. 2) held at 13C than at 2C, with or without IW. Lemons lost more weight than grapefruit (12% vs. 5.8% after 12 weeks at 13C). After removal to 20C, the fruit that had been held at 2C lost more additional weight than those that had been held at 13C. However, weight loss from fruit that had undergone IW during 2C storage was similar to that of fruit from 13C.

EC in the flavedo was lower and electrolyte leakage (total, K^+, or Ca^{2+}) was generally higher in grapefruit than in lemon (Table 1). In flavedo tissue with no visible signs of CI, there were minor significant effects between storage temperatures on EC or leakage during storage. More consistent effects were found after fruit were

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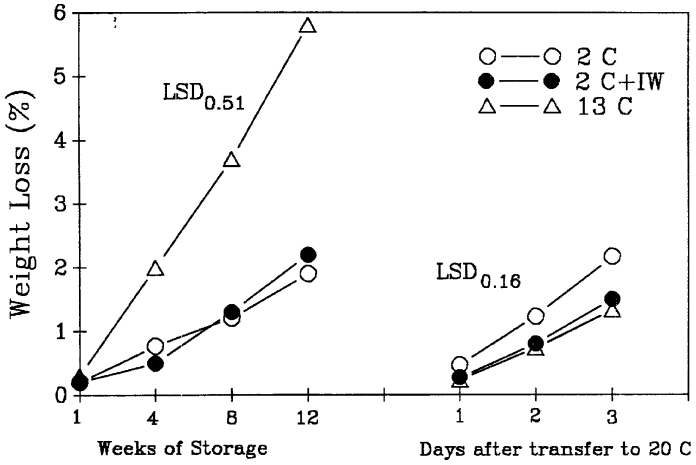

Fig. 1. Percent weight loss in grapefruit during storage under different temperature regimes and after transfer to 20C. IW= intermittent warming.

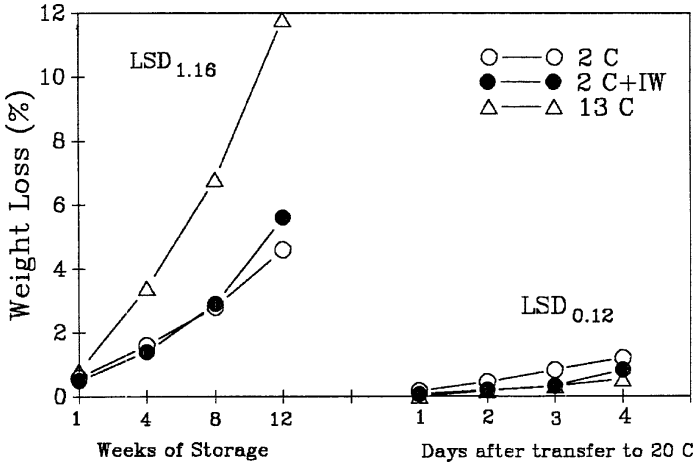

Fig. 2. Percent weight loss in lemon fruit during storage under different temperatures regimes and after transfer to 20C. IW= intermittent warming.

Table 1. Electrical conductivity (EC) and percent leakage during storage and after transfer to 20C.

	Grapefruit				Lemon				
	Weeks of storage			+3 days	Weeks of storage				+4 days
Treatment[z]	1	4	8	at 20C	1	4	8	12	at 20C
				EC flavedo (μmhos)					
13C	12.9 a[y]	12.5 a	12.8 b	18.1 b	14.6 a	15.5 a	15.4 c	17.7 a	18.1 a
2C	10.2 b	12.4 a	12.6 b	19.0 a	14.1 a	14.2 b	13.1 b	17.7 a	22.0 a
2C + IW	10.2 b	11.9 a	14.8 a	19.4 a	14.1 a	16.1 a	17.9 a	19.0 a	21.3 a
				Electrolyte leakage (%)					
13C	25.3 b	29.3 a	21.3 c	70.4 a	25.7 a	20.5 b	12.5 b	26.1 a	25.3 b
2C	30.0 a	32.1 a	23.9 b	60.4 b	19.6 b	26.6 b	14.0 b	21.7 b	26.9 b
2C + IW	30.0 a	39.5 a	31.4 a	63.0 b	19.6 b	35.4 a	21.7 a	20.6 b	32.3 a
				K^+ leakage (%)					
13C	50.6 a	71.2 a	38.5 a	48.5 b	49.3 a	58.1 a	30.9 a	46.4 a	51.6 b
2C	54.0 a	72.0 a	44.0 a	44.3 c	30.7 b	52.9 a	30.6 a	38.7 b	60.2 a
2C + IW	54.0 a	68.2 a	37.7 b	53.7 a	30.7 b	52.9 a	30.6 a	38.7 b	48.9 ab
				Ca^{2+} leakage (%)					
13C	81.9 a	92.5 a	57.7 a	73.6 b	75.2 a	91.1 a	42.4 a	84.3 a	51.0 a
2C	100.0 a	86.5 a	68.1 a	68.1 b	89.1 a	93.1 a	48.8 a	85.8 a	77.0 a
2C + IW	100.0 a	93.1 a	63.4 a	86.1 a	89.1 a	83.6 a	41.4 a	67.2 a	56.4 a

[z]IW = intermittent warming.
[y]Mean separation within columns nonsignificantly different at $P = 0.05$ according to Duncan's multiple range test.

transferred to 20C (Table 1). However, EC was significantly greater in CI-affected (pitted) tissue (24.2 and 30.9 μmhos in grapefruit and lemon, respectively) than in sound tissue (18.1 and 24.1 μmhos in grapefruit and lemon, respectively; data not shown).

The injury index in grapefruit stored at 2C was 1.1 after 6 weeks of storage, increasing to 2.6 after 12 weeks. In lemon, there was no injury after 6 weeks, whereas the rating was 2.0 after 12 weeks. Fruit that were stored at 2C with IW had reduced injury indexes of 1.1 (grapefruit) and 0.5 (lemon) after 12 weeks of storage. No CI developed on fruit held at 13C. Epidermal cells from flavedo of CI-damaged (pitted) grapefruit stored at 2C showed distinctive swollen areas with depressions (Fig. 3A) and areas of cuticular damage of the usual wax layer (Fig. 3B). Furthermore, epidermal cells from the same fruit that had been stored at 2C, but that did not yet have visible signs of CI, were revealed under SEM to be in different stages of microcollapse and cracking around the stomata (Fig. 3C), with calcium oxalate crystals growing inside the cracks (Fig. 3D). It was found that, in fruit stored at 2C with IW, the epidermal cells were complete, the stomata had no cracking around them (Fig. 3E), and the wax layer appeared uniformly smooth and porous (Fig. 3F).

Discussion

The decreased weight loss (water loss) in citrus fruit stored at 2C is probably due to the lower transpiration rate at that temperature compared with 13C. However, the water-loss rate increased dramatically once fruit were removed from 2 to 20C. McCollum (1989) also found that chilled squash fruit had a greater weight-loss rate after transfer to 15C than nonchilled fruit. We found in citrus fruit that this loss was associated with the development of microscopic cracking in otherwise sound-appearing peel tissue. Schiffmann-Nadel et al. (1980) hypothesized the existence of such cracks as an underlying cause for the development of mold in CI-affected citrus fruit. Cohen et al.(1988) made these cracks visible by dipping fruit in malachite green solution. The cuticle and outer epidermal cells of fruit that had been stored at 2C, while appearing

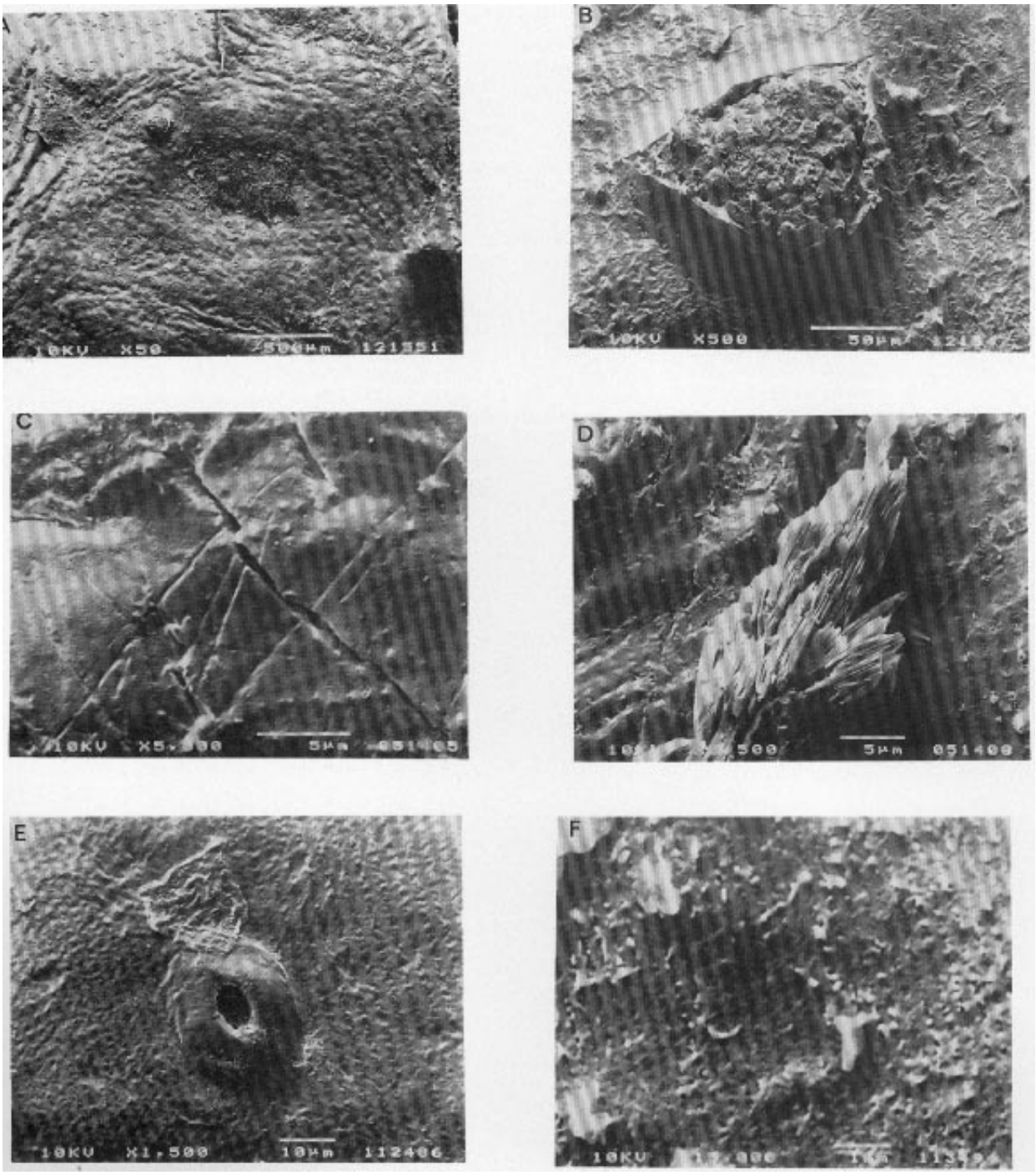

Fig. 3. Scanning electron microscopy of epidermal cells of grapefruit from chill-damaged (pitted) flavedo from fruit stored 3 months at 2C: (**A**) distinctive swollen areas, (**B**) greatly thinned wax layer. Fruit without visible damage stored at 2C: (**C**) cracking around stomata, (**D**) calcium oxalate crystals inside cracks. Fruit intermittently warmed during cold storage: (**E**) fruit surface healthy showing no cracking around stomata, (**F**) a smooth wax layer showing some wax structure.

sound, were revealed to be extensively injured. These fruit were easily infected by *Penicillium digitatum*, a wound parasite fungus that infects citrus fruit only through injuries. Our work shows that such cracks occur after exposure to chilling temperatures and are most likely sites for fungal infection.

CI symptoms can be reduced in some chilling-sensitive tissues by modifying the pattern of temperature exposure (Morris, 1982; Saltveit, 1991; Wang, 1982). IW is one such temperature modification that reduces CI in citrus (Cohen et al., 1983; Davis and Hofmann, 1973; Eaks, 1965). We report here that IW minimized water loss associated with low-temperature storage, probably by reducing the rate of cuticular cracking associated with CI. The decrease in water loss was more evident in lemon than in grapefruit, possibly because the IW formula used was developed specifically for lemon (Cohen et al., 1983).Water loss after removal from cold storage to 20C can serve as a nondestructive predictor of CI, since it was significantly (P = 0.05) correlated with CI index in grapefruit (r = 0.996) and lemon (r = 0.705).

EC and electrolyte leakage were of limited value as indicators of storage quality. Leakage increased in tissues that showed visible CI symptoms, which corresponds to the longer storage life of lemon. However, electrolyte leakage was not affected by storage

temperature, which is the most significant causal factor in CI. Our results agree with those of McCollum and McDonald (1991) that leakage is an ineffective parameter of CI in citrus and support the contention of Whitlow et al. (1992) that membrane competence cannot be effectively measured using traditional calculations of ion leakage.

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