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Essential Oils and Chilling Injury in Lemon

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Abstract. **Release, localization, and concentration of essential oils in chilling-injured and noninjured lemon [*Citrus limon* (L.) Burm.] fruit were investigated to enhance understanding of how chilling injury (CI) occurs in lemon. CI in the form of moderate to severe pitting of the flavedo was initially apparent after 3 weeks at 1 °C, followed by a gradual increase in severity until termination of the experiment after 7 weeks at 1 °C. Curing the fruit at 15 °C for 1 week before cold treatment greatly reduced the severity of CI. Release from the fruit of d-limonene, a major component of essential oil in lemon, increased with increasing amounts of CI. The enhancement of d-limonene release, however, lagged behind the development of CI. Studies of the internal anatomy of the flavedo using confocal microscopy indicated that essential oils were abundantly present inside the oil gland and in oil bodies outside the gland. Chilling-injured flavedo exhibited no obvious disruption of either the oil glands or the oil bodies. Extraction and quantification of d-limonene from chilling-injured and noninjured flavedo indicated that similar amounts of oil were present in the tissue, regardless of injury. Damage to the flavedo after 3 weeks at 1 °C was noted in the form of flattened or collapsed cells between the top of the gland and the epidermis, whereas collapse of the oil gland only was observed in later stages of injury development.**

Cold treatment can effectively disinfest citrus fruit, but application of this treatment has been hampered by sensitivity of citrus to chilling injury (CI). Chilling causes pitting and browning of the rind, which greatly reduces the marketability of the fruit.

Most of the CI is limited to the flavedo, a portion of the rind that contains large amounts of essential oils. Although these are natural components of the flavedo, they can be phytotoxic to the rind. Release of oil from damaged oil glands of the flavedo is thought to be the cause of oleocellosis, a disorder of the rind surface characterized by pebbly brown discoloration (Sinclair, 1984). This disorder also can be induced by application of essential oils onto the rind surface (Sawamura et al., 1984). If oils are released internally, they might lead to tissue collapse and the development of injury symptoms (DuPlessis, 1978).

Obenland et al. (1996) found that chilling greatly increases the release of d-limonene, the most abundant essential oil in lemons (Shaw, 1979), from the fruit surface. Increased amounts of oil release from the fruit, as estimated by changes in the amount of d-limonene emanation, may indicate an accumulation of free, potentially damaging oil within the flavedo. However, the absence of any measurements of oil release before the full development of visible rind lesions made it unclear whether the release of oil is a result or a possible cause of CI symptoms. In this study, we examined this issue by quantifying d-limonene release from lemon fruit before and during the development of CI symptoms. The anatomical localization and concentration of d-limonene in injured and noninjured areas of the rind also was determined

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Materials and Methods

Desert-grown lemons were obtained in Sept. 1995 from a commercial packing plant in Yuma, Ariz. Green fruit picked early in the day that were not washed or waxed were selected out of bins containing fruit from a single grower. The fruit was held at 25 °C in the packing plant before selection. Fruit sizes ranged from 140 to 165 fruit per box (85 to 140 g/fruit). The fruit were transported to Fresno in a van kept at 21 °C. Upon arrival at Fresno, the fruit were stored at 20 °C and 20% relative humidity (RH) before treatment the following day. Separate, randomly selected batches of fruit from this single lot were considered to be replications.

Cold treatment consisted of storing the fruit at 1 °C for 1, 3, 5, or 7 weeks. Fruit stored at 10 °C was used as the control. To observe the effect of curing on rind injury and d-limonene release, a portion of the fruit was kept for a week at 15 °C and 80% RH before the initiation of cold treatment. Following the cold or control treatments the fruit was kept for 1 week at 20 °C for development of CI symptoms.

Quantification of d-limonene release was performed by headspace measurement and solid phase microextraction as described by Obenland et al. (1996). Ten lemons were used per measurement and each measurement was replicated three times using different lemons. The fruit was at 20 °C for all measurements.

After determination of d-limonene release, all fruit (10 fruit per replication) were visually evaluated individually for rind injury and placed into one of six numerically weighted classes based on the size and number of lesions on the fruit: healthy (0), very slight (1), slight (2), moderate (4), severe (8), and very severe (16). Each fruit was scored, the results from each fruit summed, and the resulting number divided by the total number of fruit. Following injury rating, 7-mm-diameter disks were taken from injured and noninjured areas of the rind, the albedo was trimmed away from the disks, and the resulting flavedo samples were frozen for later d-limonene extraction.

To study anatomical structure of the flavedo and localization of the oil, sections 25 mm long and 2.5 mm thick were excised from the flavedo in visibly injured and noninjured areas of the rind of chilling-injured lemons. Sections were placed on a slide and stained with Nile red at 0.01 $\mu g \cdot \mu L^{-1}$ (Sigma Chemical Co., St. Louis) to label lipids and other hydrophobic compounds. A glass coverslip was then fixed on top of the sample using clear adhesive tape. The section was examined with confocal microscopy at ×10 using a Leica TCS-4D microscope (Leica, Heidelberg, Germany) with an excitation of 488 nm and dual detectors with barrier filters of 515 nm (long pass) and 590 (long pass). It was apparent that even areas of the flavedo that were severely pitted as a result of cold treatment displayed little obvious anatomical change; thus, fruit from another lot that had been kept at 1 °C for a longer period (12 weeks) was examined as a comparison.

D-limonene was extracted from flavedo samples using supercritical fluid extraction (SFE). Before extraction, one frozen flavedo disk was immersed into liquid N for 10 s to make the sample brittle. The flavedo disk was then quickly removed from the liquid N, placed within a folded sheet of filter paper, and crushed with a hammer to disrupt the tissue matrix. This step was followed by rapidly inserting the sample and filter paper into an extraction thimble, filling the remaining void volume with tissue paper, and sealing the thimble. Extraction was accomplished using an HP7680T supercritical fluid extractor (Hewlett-Packard, Avondale, Pa.) and SFC grade CO_2 (Matheson, Newark, Calif.) as the extraction fluid. Density of the extraction fluid was maintained at 0.25 $g \cdot mL^{-1}$ during the extraction using a pressure of 7700 kPa and a chamber at 40 °C. Following a 5-min equilibration, the samples were extracted for 16.7 min using a flow rate of 1.5 $mL \cdot min^{-1}$. Analytes were collected at 0 °C on a Hypersil ODS trap that was standard equipment for the instrument. To elute analytes, the trap temperature was increased to 40 °C and ethanol was passed through at 1 $mL \cdot min^{-1}$. After removal of analytes, the trap was again cooled and another complete extraction of the flavedo disk was performed. Analysis of the extracted components for d-limonene was performed using gas chromatography/mass spectroscopy as described by Obenland et al. (1996), except that the injector port temperature was decreased to 250 °C. The results from the two

extractions per disk were combined. For each treatment, an extraction was performed on flavedo tissue from five lemons using individual extractions of three flavedo disks per lemon. Extraction data were tested with an analysis of variance (ANOVA) and by means separation (Fisher's Protected least significant difference) using SuperANOVA (Abacus Concepts, Berkeley, Calif.).

Results

CI symptoms were readily apparent in the noncured fruit after 3 weeks at 1 °C, with most of the fruit showing moderate to severe rind injury (Fig. 1A). This injury was characterized by extensive pitting of the flavedo (Fig. 2). Cured fruit given the same treatment showed only slight damage after the same interval at 1 °C. By 5 and 7 weeks of storage at 1 °C, most of the noncured fruit were rated as being severely or very severely damaged. However, even after 7 weeks of cold storage only a slight to moderate amount of injury was observable in the cured fruit. Rind damage was absent for the duration of the experiment for fruit stored at 10 °C.

The pattern of d-limonene release from the fruit (Fig. 1B) diverged from that of symptom development (Fig. 1A). Release of d-limonene from the noncured fruit increased only slightly after 3 weeks at 1 °C as compared to 1 week, the amount detected being similar to that before storage. This result contrasts with the large increase in visible rind injury between 1 and 3 weeks. Release did not begin to increase to any extent from the noncured fruit until after 5 weeks of storage where the amount of d-limonene detected increased by 3 times over the amount after 3 weeks of storage. The amount of d-limonene given off by the fruit after 7 weeks of storage was even higher. For cured fruit, there was little or no d-limonene detectable following cold treatment until at 7 weeks of storage, when the quantity of d-limonene detected had begun to increase. The same pattern was observed for fruit stored at 10 °C, with the exception that a lesser amount of d-limonene was detected at 7 weeks.

Confocal microscopy was used to compare injured regions of cold-treated fruit with areas of the same fruit that showed no apparent injury (Fig. 3 A and B). Injured flavedo tissue showed areas of flattened or collapsed cells immediately above the oil glands. Collapse of the oil gland was not commonly observed in injured areas of fruit kept at 1 °C for 3 weeks but was present in tissues that had undergone more extensive cold treatment (Fig. 3C). Oil fluoresced strongly after staining with Nile red and was easily visible. Oil occupied only a small portion of the oil glands in injured and noninjured tissue. Large amounts of oil were also present outside of the glands in the form of discrete oil bodies in injured and noninjured tissue.

Comparison of injured and noninjured areas from the same lemons stored for 7 weeks at 1 °C indicated that the injured areas released 4 times as much d-limonene as the noninjured areas (data not shown). Thus, the injured areas were most responsible for the d-limonene emission values measured for whole fruit. To determine if injured areas showed any significant depletion of d-limonene during storage relative to uninjured areas, d-limonene was extracted and quantified from both areas (Table 1). The amount of d-limonene present in the tissues was similar in all of the tissue types extracted. Injured flavedo portions after 3 and 5 weeks of storage at 1 °C were, however, statistically different from each other. To answer the question of whether or not essential oils enter the normally oil-free tissue of the albedo during CI, albedo immediately beneath severely injured areas also was extracted. No d-limonene was ever detected from this tissue.

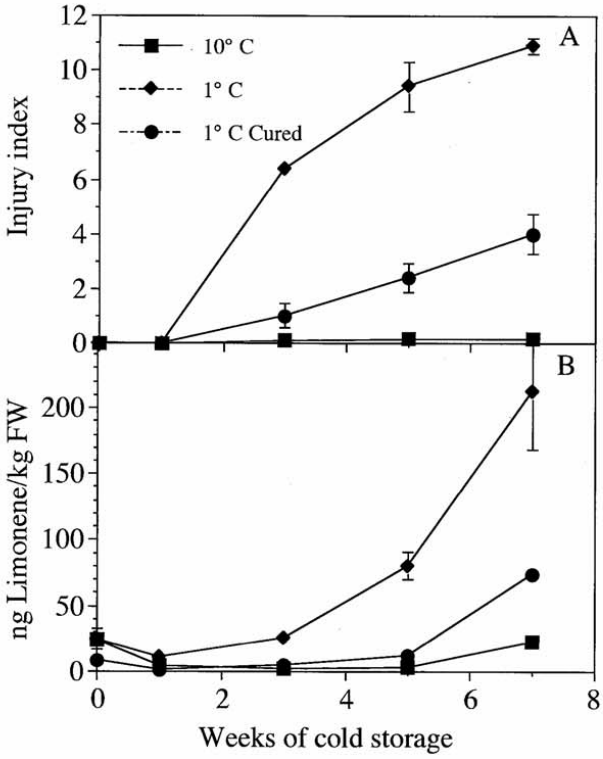

Fig. 1. (**A**) Rind injury and (**B**) d-limonene release from lemons stored at 1 or 10 °C for various lengths of time. Error bars indicate standard error and bars not visible are covered by symbol.

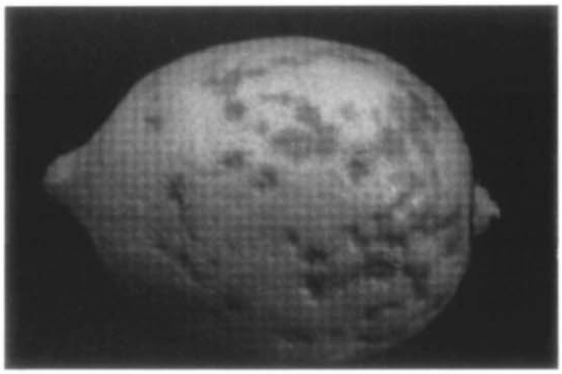

Fig. 2. Chilling injury on lemon rind after 3 weeks at 1 °C followed by 1 week at 10 °C.

Discussion

The release of d-limonene from lemon fruit increased as CI symptoms became more severe (Fig. 1). Curing greatly reduced CI, as had been also observed previously (Chalutz et al., 1985; Hatton and Cubbedge, 1982; Houck et al., 1990); as a result, cured fruit released far less d-limonene than did noncured fruit over the course of the experiment. However, while CI and release of d-limonene were related, d-limonene release lagged behind the development of injury. This difference was especially apparent after 3 weeks of cold storage at which time moderate to severe CI was present in the majority of the fruit and yet little or no increase in d-limonene release from the fruit was detectable. This result raises the prospect that CI is the cause of the increased release of d-limonene (and other essential oils) and that essential oils are not a primary causal factor in the development of CI. However, our measurements do not exclude the possibility that damage-causing essential oils are being released internally in the flavedo but that the release is not adequately reflected by headspace measurements until damage becomes severe

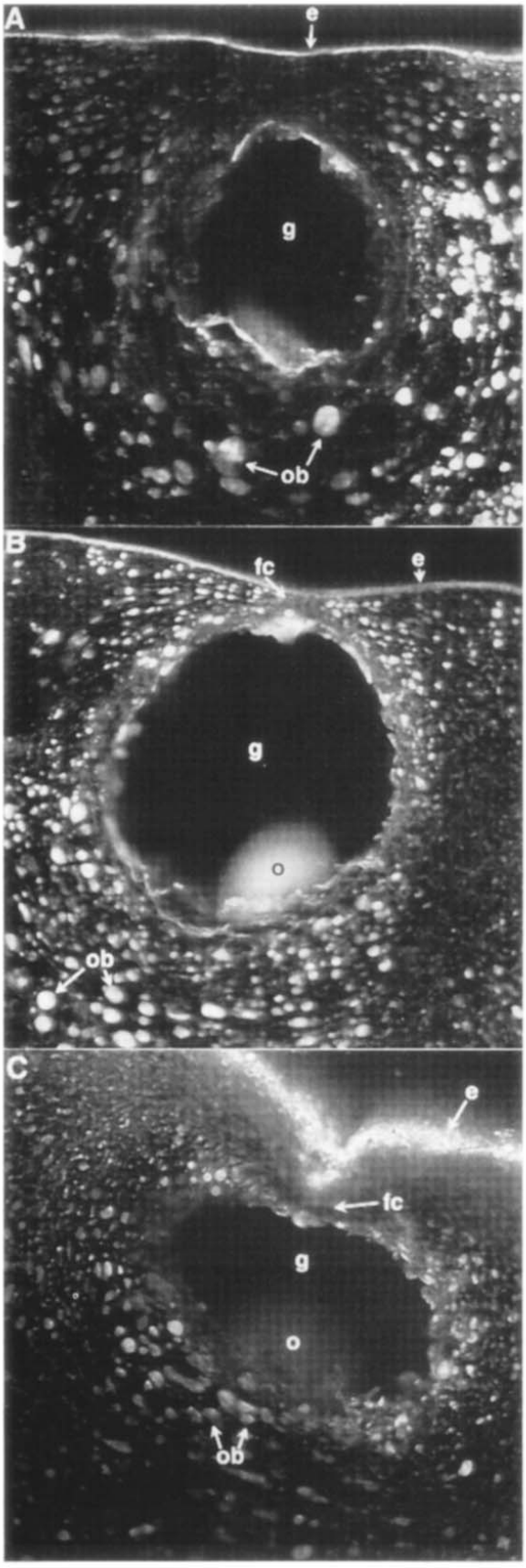

Fig. 3. Cross sections of oil glands and surrounding tissue from chilling-injured and noninjured flavedo, (×10). (**A**) Noninjured flavedo from a lemon displaying chilling injury. Lemon had been stored at 1 °C for 3 weeks followed by 10 °C for 1 week. (**B**) Injured flavedo from a chilling-injured lemon. Lemon was the same as was used for (**A**). (**C**) Severely injured flavedo from chilling-injured lemon that had been stored at 1 °C for 12 weeks. Epidermis (**e**), flattened cells (**fc**), gland (**g**), oil (**o**), oil body (**ob**).

enough to allow an easier diffusion of the oil out of the rind.

Using confocal microscopy with fluorescence, localization of essential oils in chilling-injured and noninjured flavedo was visible without fixing the tissue or mechanically sectioning through the glands. This procedure allowed visualization of the essential oils under more natural conditions than possible with other types of microscopy and enabled us to further explore the question of whether or not essential oils are being released within the flavedo during the development of CI. While most attention has been placed on the oil gland as the site of accumulation of the essential oils in citrus flavedo (Bosabalidis and Tsekos, 1982; Shomer, 1980), it was apparent that a sizeable proportion of the essential oils reside in bodies exterior to the gland (Fig. 3). This observation may be important because we found the oil gland to be much more resistant to collapse than surrounding cells and, therefore, perhaps less likely to be damaged and less able to release phytotoxic essential oils into flavedo tissues as a result of chilling damage. Although oil gland collapse has been previously reported to be a symptom of chilling damage (Underhill et al., 1995), we typically saw no oil gland collapse until later stages of CI. Comparisons of injured and noninjured flavedo sections did not reveal a discernible disruption of the oil-bearing bodies or of the oil glands after 3 weeks of cold treatment even though the fruit was severely pitted. This pattern corresponds to the finding of little or no differences in the d-limonene content extractable from these tissues (Table 1). Most of the essential oils in the flavedo before injury remain there localized even in extensively injured tissue (Fig. 3C). Cells between the epidermis and the top of the oil glands did, however, show flattening and collapse as a result of injury. This collapse appears to be responsible for the pitting visible on the rind surface. Oil bodies present in this area of the flavedo may have undergone disruption during collapse, causing a release of oil into surrounding cells.

Results from this experiment do not support a role for essential oils as a primary cause of CI in lemons. Essential oils, however, are clearly being released in far greater amounts from chilling-injured flavedo. Considering the highly phytotoxic nature and large abundance of essential oils in the flavedo, it seems likely that the essential oils may have some responsibility for the development of CI. Further detailed study of the biogenesis of essential oils and their compartmentalization would be useful in ascertaining their involvement in lemon CI.

Literature Cited

Bosabalidis, A. and I. Tsekos. 1982. Ultrastructural studies on the secretory cavities of *Citrus deliciosa* Ten. II. Development of the essential oil-accumulating central space of the gland and process of active secretion. Protoplasma 112:63–70.

Chalutz, E., J. Waks, and M. Schiffmann-Nadel. 1985. Reducing susceptibility of grapefruit to

Table 1. Concentration of d-limonene in injured and noninjured lemon flavedo.

Temp (°C)	Storage time (weeks)	Injury	D-limonene extracted ($\mu g \cdot cm^{-2}$)
---	0	---	10.50 (±1.70)
10	3	---	11.20 (±1.03)
1	3	---	10.49 (±1.03)
1	3	+	13.05 (±1.16)*
1	5	---	10.54 (±0.85)
1	5	+	9.26 (±0.60)*

*Values marked with asterisk differ significantly from each other ($P \leq 0.05$).

chilling injury during cold treatment. HortScience 20:226–228.

DuPlessis, D.S.F. 1978. Skilprobleme by sitrus: Oleocellose in gepokte skil. Citrus & Subtropical Fruit J. 534:12–16.

Hatton T.T. and R.H. Cubbedge. 1982. Conditioning Florida grapefruit to reduce chilling injury during low-temperature storage. J. Amer. Soc. Hort. Sci. 107:57–60.

Houck, L.G., J.F. Jenner, and B.E. Mackey. 1990. Seasonal variability of the response of desert lemons to rind injury and decay caused by quarantine cold treatments. J. Hort. Sci. 65:611–617.

Obenland, D.M., D.C. Fouse, L.H. Aung, and L.G. Houck. 1996. Release of d-limonene from non-injured and injured lemons treated with hot water and low temperature. J. Hort. Sci. 71:389–394.

Sawamura, M., T. Manabe, K. Oonishi, K. Yashuoka, and H. Kusunose. 1984. Effects of rind oils and their components on the induction of oil spot in citrus species. J. Hort. Sci. 59:575–579.

Shaw, P.E. 1979. Review of quantitative analysis of citrus essential oils. J. Food Agr. Chem. 27:246–257.

Shomer, I. 1980. Sites of production and accumulation of essential oils in citrus fruits. Electron Microscopy 2:256–257.

Sinclair, W.C. 1984. The biochemistry and physiology of the lemon and other citrus fruits. Univ. of California, Div. of Natural Resources, Oakland.

Underhill, S.J.R., R.L. McLauchlan, and I.L. Eaks. 1995. 'Eureka' lemon chilling injury. HortScience 30:309–312.