was out of synchronization with healthy trees (8) and was
greater at the time of the 1982 freeze than in trees infected
with a mild isolate or in healthy trees. The lack of similar,
treatment-specific damage in the severe cold of 1981 suggests
that a specific set of circumstances was involved.
The degree of bark scaling observed apparently also is
freeze correlated. Severe CEV-induced bark scaling is un-
common in young, recently infected Carrizo citrange trees.
Although the bark scaling observed here is obviously CEV-
treatment associated, it seems likely it was enhanced by the
freeze, which may have amplified the cytopathological
changes in the cortex associated with CEV infection (7).
The similar cold injury pattern observed in the single
set of 'Valencia'/trifoliate orange trees suggests that the in-
jury effect was not scion- or stock-specific, but a more
thorough comparison of this point is needed. Lack of CEV-
specific injury in other plots with 'Valencia' scions on
Carrizo stock could suggest that a scion effect is involved.

Regardless of the actual mechanism involved, the injury
observed deserves consideration in the development of tree-
dwarfing strategies based on use of CEV. Not all CEV iso-
lates tested were associated with cold injury, but most iso-
lates which cause a strong citron reaction and bark scaling
on trifoliate orange were. Since CEV-induced dwarfing is

more pronounced in citranges than trifoliate orange (1, 4, 9),
use of fairly strong isolates of CEV may be required to
achieve marked dwarfing of trees on Carrizo rootstocks. Use
of such strong isolates in cold areas would seem to entail
increased risk of cold injury, at least under some conditions.
Further tests on viroid, scion, stock, and climatic interaction
are needed.

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INDICATORS OF CITRUS COLD-HARDENING IN THE FIELD

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logical factors.

Abstract. Several physiological factors in leaves of 'Va-
lenca' orange (Citrus sinensis (L.) Osb.) trees on sour orange
(C. aurantium L.) rootstock were periodically measured in a
3-year-old planting in central Florida during 22 consecutive
months. Leaves were picked just before sunrise and com-
posite samples were subdivided for different analyses. Sugar
and proline concentrations, water content, and osmotic po-
tential of expressed sap were found correlated with average
air temperatures between sampling dates and with LTo
values after freeze tests in controlled-temperature facilities.
Lesser associations with temperature were found for water-
soluble proteins, amino acids (minus proline), starch, ATP
levels, and xylem-pressure potentials. The concentration of
expressed sap from the leaves was considered one of the
more promising factors to index cold hardening in practical
citriculture.

Tree-environment relationships are becoming increas-
ingly important in citriculture as production costs continue
to increase. One major area of concern is freeze protection.
Satellite freeze warning systems (8) help to alert growers to
impending damaging freeze conditions, and broad tempera-
ture guidelines (15) help to identify critical temperatures
for trees and fruit. These guidelines largely apply to general
rather than specific situations where growers are confronted
with different cultivars and different degrees of cold hardi-
ness. Indices of citrus cold hardening in the field are mostly
limited to bark peeling (22, 25), determinations of leaf
freezing points (9, 21), and nonvisible growth (20). All of
these help to reinforce growers' experiences toward making
decisions during freeze situations. But there are serious draw-
backs in all three of the approaches.

In our work on seasonal trends of physiological factors
in leaves of citrus trees (19), temperature relationships with
some of the factors studied suggest additional approaches
toward indexing cold hardiness in the field. These tempera-

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nmission of exococcus dwarfing factors into Washington navel orange
ture relationships with physiological factors in citrus leaves are presented in this report.

Materials and Methods

Citrus trees. Trees, arbitrarily selected for study, were 3-yr-old 'Valencia' sweet orange (Citrus sinensis (L.) Osb.) grafted on 4-yr-old sour orange (C. aurantium L.) rootstock in a 98-tree research planting in central Florida. Budwood was from a single healthy sweet orange tree that had been indexed and found free of psorosis, exocortis, and xylemoprosis viruses.

Trees received no special care beyond regular grove practices in fertilization, insect and disease control, and watering with overhead sprinklers to prevent visible water stress during dry periods. A hygrothermograph, accurate to 1°F, recorded air temperatures in a standard weather shelter 4 1/2 ft above ground level. Biochemical and physical determinations were made on leaves from 5 single-tree replicates.

Sampling. Samples of 60 leaves per tree consisted of 15 mature leaves of approximately equivalent age from each of the 4 cardinal sides, 2 to 6 ft above ground level. Leaves were sampled within the hour before sunrise about every 3 wk for 22 continuous months. Samples were transported in ice-cooled, plastic bags to laboratory facilities 30 min away.

Physiological factors. Leaves were sponge-cleaned, blotted dry, and partitioned for different analyses. Ten leaves were used to sequentially determine water content, carbohydrates, and amino acids. Ten leaves were also used to determine water-soluble proteins and ATP. Three leaves were used to determine xylem-pressure potential and 20 leaves for expressed-sap and osmotic potential. The remaining 17 leaves were used in leaf-freezing tests for cold hardness determinations. Biochemical determinations were duplicated.

Water content. Ovendry procedures (11) were used to dry leaf tissues overnight, after which water content was calculated.

Carbohydrates. Carbohydrates in ovendried leaves ground in a Wiley mill were extracted with 76% (v/v) ethanol in a Soxhlet extractor. Impurities were removed with ion-exchange resins, and sugars were determined by the anthrone and Somogyi-Nelson methods outlined by Hodge and Hofreiter (4). Sucrose content was calculated as total sugar content minus reducing sugars.

Starch in the ethanol-insoluble residue was solubilized in boiling water for 15 min and then extracted by the method of MacRae (7). The resultant glucose was purified with ion-exchange resins and quantitated with glucose oxidase reagent.

Amino acids. Free amino acids were part of the initial extraction from finely ground, ovendried leaves in 76% (v/v) ethanol for 6 hr in a Soxhlet extractor. Ethanol was evaporated and the residue taken up in glass-distilled water. The water fraction was passed through Dowex 50-8X, 100-200 mesh ion-exchange columns, and the amino acids were eluted with 4N NH₄OH. Total amino acids and proline were determined by ninhydrin procedures (5, 10, 14).

Proteins. Water-soluble proteins were extracted from finely ground, freeze-dried leaves with mortar and pestle. Minimum volume of 0.5 ml of 0.1 M phosphate buffer (pH 7.2) containing 0.25 M sucrose, 1mM dithiothreitol, and 0.5 g of polyvinylpyrrolidone was used per gram of dry tissue. Soluble proteins were separated from cell debris by centrifugation at 12,000 g for 30 min. Additional purification included dialysis overnight and trichloroacetic acid precipitation prior to determining proteins by the Lowry method (6).

Adenosine triphosphate (ATP). ATP was extracted from finely ground, freeze-dried leaves in boiling water (1). Leaf samples were not exposed to light prior to freeze-drying. ATP was determined according to St. John (12).

Xylem-Pressure and osmotic potential. Xylem-pressure potential of leaves was determined by a pressure-bomb technique (2). Osmotic potential was determined in a freezing-point osmometer on sap from leaves pressed with 2 metric tons after the leaves had been cleaned, surface-dried, and thoroughly frozen (5).

Cold hardiness. Cleaned but surface-wet leaves were placed side by side in thin, polyethylene wraps suspended in temperature-controlled freeze facilities. The wet leaf surfaces prevented supercooling, and leaf temperatures inside polyethylene wraps were within ± 0.5°F of ambient air. Freeze tests started at 28°F, and thereafter the temperature was abruptly decreased 2°F every 2 hr. Frozen leaves were removed at 2-hr intervals after the start of the test until 4 samples were collected (i.e., 28°, 26°, 24°, and 22°F samples). One-half of the leaves removed were placed under mist in a greenhouse and, after 36 hr, rated for freeze injury on the amount of permanent water soaking, and calculated for mean lethal temperature for 50% kill (LT₅₀).

Results and Discussion

Physiological factors that markedly increased in concentration in leaves of citrus during cooler temperatures in partially controlled-environment studies (17, 18) also increased during cooler temperatures in the field. The concentrations of sugars and proline correlated well (coefficients (r) greater than 0.8) with average temperatures between sampling dates in this study (Fig. 1). Concentrations of total sugars increased from about 45 mg/g dry weight at 77°F average air temperature to more than 90 mg/

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Fig. 1. Correlation (R) of total sugars (a) and proline (b) in 'Valencia' orange leaves with average air temperatures between sampling dates near Leesburg, FL.

at 50°F. The rate of increase averaged about 1.5 mg per 1° decrease between 77° and 59°F, and almost doubled to about 2.9 mg/1°F decrease between 59° and 50°F average air temperatures. The temperature relationship with proline was more linear than with sugars. Proline concentrations increased from about 4 mg/g dry weight at 77°F to 10 mg at 50°F, rate of about 0.2 mg/1°F decrease.

Water content of the leaves and the concentration of expressed sap were two other factors that correlated better than 0.8 with average field temperatures (Fig. 2). The observation of decreasing water content with cooler temperatures under apparent non-water-stress field conditions suggests changes not only in stomatal behavior but also in hydraulic resistance and water uptake through citrus roots, which have been studied by others (15, 16). The decrease in water content per 1°F change is relatively very small at less than 0.02 g/g dry weight, but 1.4 g of water per gram of leaf dry weight at 50°F average air temperature is in line with observations of increased cold hardiness in citrus leaves (18). Decreases in water content with concomitant increases in sugars and proline probably contributed the most to increases in expressed sap concentrations, which also correlated well with average air temperatures in this study. The increases in sap concentration amount to 2° to 3° F, at the most, in freezing point depression which would help in freeze avoidance.

The other factors studied were poorly correlated with field temperatures. Correlation coefficients were less than

Table 1. Correlation (r) of physiological factors in 'Valencia' orange leaves with average field temperatures between sampling dates near Leesburg, FL, and determinations of lethal temperature for 50% kill (LT50) in leaves freeze-tested at controlled temperatures.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Temp (°F)</th>
<th>LT50 (°F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sugars</td>
<td>-0.894</td>
<td>-0.733</td>
</tr>
<tr>
<td>Proline</td>
<td>-0.847</td>
<td>-0.754</td>
</tr>
<tr>
<td>Water content</td>
<td>0.885</td>
<td>0.860</td>
</tr>
<tr>
<td>Sap concn</td>
<td>0.801</td>
<td>0.825</td>
</tr>
<tr>
<td>Starch</td>
<td>0.273</td>
<td>0.149</td>
</tr>
<tr>
<td>Amino acids (minus proline)</td>
<td>-0.037</td>
<td>-0.188</td>
</tr>
<tr>
<td>Water-soluble proteins</td>
<td>-0.348</td>
<td>-0.171</td>
</tr>
<tr>
<td>Xylem-pressure potential</td>
<td>0.326</td>
<td>0.040</td>
</tr>
<tr>
<td>ATP (adenosine triphosphate)</td>
<td>-0.005</td>
<td>-0.202</td>
</tr>
<tr>
<td>Temp (°F)</td>
<td></td>
<td>0.866</td>
</tr>
</tbody>
</table>

0.4 for xylem-pressure potentials, starch content, amino acids (minus proline), water-soluble proteins, and ATP concentration (Table 1).

In controlled-temperature studies, LT50 values are used to index cold hardiness in citrus (22). In this study, LT50 determinations on detached leaves in controlled freezes were significantly correlated with average air temperatures, sugars, proline, water content, and increases in sap concentration (Table 1). These apparent relationships found between physiological factors and field temperatures as well as between physiological factors and LT50 values, plus past observations in controlled-environment studies (17, 18), support the contention of utilizing field temperatures and/or physiological factors to index cold hardening of citrus trees in the field. However, the variability of the data in Figs. 1 and 2, as well as those in Figs. 3, 4, and 5 demonstrates the extensive work needed to approach the ± 1°F accuracy considered critical in making grove management decisions during damaging freezes in Florida. The concentration of expressed sap and/or field temperatures are likely candidates for research and development toward indexing citrus cold hardiness in the field largely because of convenience, ease, and overall cost.

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