Sugar utilization by citrus juice cells as determined by $[^{14}\text{C}]$-sucrose and $[^{14}\text{C}]$-fructose feeding analyses

Raphael Goren$^{a}$$^{*}$, Moshe Huberman$^{a}$, Uri Zehavi$^{b}$, Malka Chen-Zion$^{a}$, Ed Echeverria$^{c}$

$^{a}$ Kennedy-Leigh Centre for Horticultural Research, The Hebrew University of Jerusalem, POB 12, 76100 Rehovot, Israel
$^{b}$ Institute of Biochemistry, Food Sciences and Nutrition, The Hebrew University of Jerusalem, Rehovot, Israel
$^{c}$ Citrus Research and Education Center, The University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA
$^{*}$ Author to whom correspondence should be addressed (fax +972 8 9489574; e-mail r gore@agri.huji.ac.il)

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Abstract – Sugar utilization by mature citrus juice cells was investigated in light of previous reports suggesting the inability of these cells to phosphorylate hexoses. Grapefruit juice sac cells were incubated in solutions of $[^{14}\text{C}]$-sucrose or $[^{14}\text{C}]$-fructose for 16 h during which $[^{14}\text{CO}_2]$ evolution was measured by trapping into soluene. After the incubation period, tissue was extracted in 5% trichloroacetic acid or 8% ethanol and extracts separated and identified by thin layer chromatography. Fructose was taken up and metabolized more rapidly than sucrose. In both cases, significant amounts of $[^{14}\text{CO}_2]$, $[^{14}\text{C}]$-pyruvic and $[^{14}\text{C}]$-citric acid were recovered after incubation. In separate experiments, hexokinase activity in tissue extracts was found to co-sediment with mitochondrial fractions but was not detected in the soluble fractions as previously reported. The data indicated that, contrary to earlier observations, mature citrus fruit juice cells contain the enzymatic machinery to metabolize soluble sugars. This is consistent with the glycolytic utilization of sugars in cells undergoing anaerobic respiration. © 2000 Éditions scientifiques et médicales Elsevier SAS

Citrus / fructose / hexokinase / phosphofructokinase / sucrose / sugar utilization

ATP, adenosine 5'-triphosphate / DTT, dithiothreitol / EDTA, ethylenediamine tetraacetaete / EtOH, ethanol / GC, gas chromatography / HPLC, high performance liquid chromatography / MES, 2-[N-morpholino]ethanesulfonic acid / NADP, nicotinamide-adenine dinucleotide phosphate / PFK, ATP-dependent phosphofructokinase / PFP, pyrophosphate-dependent phosphofructokinase / PPI, inorganic pyrophosphate / PSi, pounds per square inch / PVP, polyvinyl phosphate / TCA, three chloroacetic acid / TLC, thin layer chromatography

1. INTRODUCTION

The respiratory carbon source in harvested citrus fruits has remained largely unidentified as a result of several pieces of evidence pointing to the inability of juice cells to utilize simple sugars and supporting their utilization of organic acids. First, the concentration of soluble sugars in citrus fruit continues to increase even after harvest [13, 16] despite the absence of starch or other reserve polysaccharides. Second, no hexokinase activity was detected in protoplasts prepared from fresh citrus fruits that otherwise were able to degrade sucrose [14]. Third, citrate, a potent inhibitor of citrus inorganic pyrophosphate (PPI)-dependent phosphofructokinase (PFP) [24], was reported to be present in the cytosol at concentrations high enough to inhibit this enzyme [14]. Fourth, feeding experiments with $[^{14}\text{C}]$-citrate demonstrated that organic acids are rapidly utilized for respiratory purposes [20], a finding that coincides with the decline of citrate in harvested citrus [7, 13, 18, 22].

More recently however, using a more precise nonaqueous cell fractionation procedure [24], we demonstrated the absence of citrate in the cytosol of citrus juice cells and the presence of hexokinase in stored citrus fruits [15]. We also found that the concentrations of cellular fructose-2,6-biphosphate found in citrus juice cells are sufficiently high to activate PPI-dependent PFP [24]. These findings and reported demonstrations of enhanced PFP activity in maturing fruit cells [2] and plant cells under stress [6, 10] led us to re-examine the possible role of sugars as a carbon source in harvested...
Table 1. Release of $^{14}$CO$_2$ and the radioactivity in tissues extracted with either trichloroacetic acid or ethanol from grapefruit juice sacs. Juice sacs were incubated with $[^{14}$C]-glucose, $[^{14}$C]-fructose or $[^{14}$C]-sucrose for 16 h. Values are means ± SE of four to six experiments. $^{1}$ Organic acid fraction before analysis by TLC and HPLC (Methods section 4.8).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Medium (dpm)</th>
<th>$^{14}$CO$_2$ (dpm)</th>
<th>Uptake (dpm)</th>
<th>Uptake (%)</th>
<th>% Residue (dpm)</th>
<th>% Residue (%)</th>
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<tr>
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<td>(dpm$^{-1}$)</td>
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<tr>
<td>TCA extraction</td>
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<tr>
<td>$[^{14}$C]-Sucrose</td>
<td>5.612</td>
<td>3.396 ± 616</td>
<td>530 ± 74</td>
<td>9.49 ± 1.01</td>
<td>1.861 ± 409</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>$[^{14}$C]-Fructose</td>
<td>5.582</td>
<td>7.839 ± 666</td>
<td>510 ± 37</td>
<td>9.14 ± 0.32</td>
<td>5.450 ± 606</td>
<td>1.07 ± 0.11</td>
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<tr>
<td>EtOH extraction</td>
<td></td>
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<tr>
<td>$[^{14}$C]-Glucose</td>
<td>5.410</td>
<td>10.265 ± 814</td>
<td>421 ± 30</td>
<td>7.78 ± 0.30</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$[^{14}$C]-Sucrose</td>
<td>5.410</td>
<td>3.317 ± 50</td>
<td>380 ± 13</td>
<td>7.37 ± 0.30</td>
<td>2.700 ± 130</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>$[^{14}$C]-Fructose</td>
<td>5.633</td>
<td>7.700 ± 300</td>
<td>378 ± 25</td>
<td>7.20 ± 0.15</td>
<td>6.067 ± 160</td>
<td>1.61 ± 0.12</td>
</tr>
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</table>

citrus fruits. The results of feeding experiments reported here demonstrate the presence of hexokinase activity in citrus juice cells and their ability to utilize sugars (sucrose and hexoses) through glycolysis and the tricarboxylic acid cycle.

2. RESULTS

2.1. $[^{14}$C]-Glucose, $[^{14}$C]-fructose and $[^{14}$C]-
sucrose feeding experiments

When juice sacs were incubated in either $[^{14}$C]-
sucrose or $[^{14}$C]-fructose for 16 h, between 7 and 9 % of the total radioactivity was taken up by the cells. Radioactivity in the trapped CO$_2$ was equivalent to between 0.6 and 1.5 % of the total sucrose or fructose taken up in the trichloroacetic (TCA) extracts, whereas in the ethanol (EtOH) extracts, the amount of $^{14}$CO$_2$ trapping was 0.9 and 2.0 % for $[^{14}$C]-sucrose and $[^{14}$C]-fructose, respectively (table 1). After each incubation period, juice sac tissues were extracted with two distinct extraction procedures in order to verify the results (see Methods). The remaining radioactivity in tissue extracts was measured after partial purification by fractionation either with ethyl acetate (following the TCA extraction) or by running through a Dowex-1 column after the EtOH extraction (table 1). It is interesting to note that although the supplied radioactivity in the incubation media was similar for both sugars, $^{14}$CO$_2$ evolution and residual radioactivity were more than two-fold higher after incubation with $[^{14}$C]-fructose than incubation with $[^{14}$C]-sucrose. Similar results were obtained when $^{14}$CO$_2$ evolution was measured after incubation with $[^{14}$C]-glucose compared to that obtained after incubation with $[^{14}$C]-
sucrose. The higher proportion of radioactivity in the residue fraction of the EtOH extraction than that obtained after the TCA extraction can probably be attributed to the differences in extraction methods (table 1).

Extraction in TCA and fractionation with ethyl acetate offers the advantage of allowing separation between weaker and stronger organic acids (such as pyruvic and citric acids). The acid extract was examined by thin layer chromatography (TLC) using two developing solvents (see Methods). Most of the radioactivity was identified at an R$_f$ value of 0.5–0.6 which coincides with standard $[^{14}$C]-pyruvic acid. Similar results were obtained for both developing solvents (figure 1). Recovery test showed that more than 90 % of labeled pyruvic acid could be recovered at the end of the extraction and purification steps indicating that $[^{14}$C]-pyruvic acid was stable.

The EtOH extraction procedure yielded both pyruv-
ic and citric acids. The extract media were analyzed by high performance liquid chromatography (HPLC) after their partial purification on the Dowex-1 column. More than 90 % of total radioactivity (of the $[^{14}$C]-pyruvic and $[^{14}$C]-citric acids standards) could be eluted by 4 M formic acid (figure 2). To determine the R$_f$ values for citric and pyruvic acids, a mixture of standard carboxylic acids was separated by HPLC (citric acid at R$_f$ 14.6 and pyruvic acid at R$_f$ 17.8 and 18.4; figure 3). In the experimental tissue extracts, most of the radioactivity (representing several organic acids) was found in tubes 8 to 10 (figure 4 A, B). Citric and pyruvic acid markers were identified in tubes 8 and 9, respectively (figure 4 C, D). Increasing the incubation time of the juice sacs up to 16 h resulted in more radioactivity in other R$_f$ fractions. Attempts to separate these two acids from accompanying organic acids by HPLC were not satisfactory. A large amount of $[^{12}$C]-citric acid mixed with $[^{14}$C]-citric acid was also detected in tube 9, where it masked the $[^{14}$C]-
Sugar utilization by citrus juice cells

Figure 1. Thin layer chromatogram of organic acids from partially purified juice sacs extracted with TCA. Juice sacs were incubated with $^{14}$C-sucrose or $^{14}$C-fructose for 12 h and extracted with TCA (see table I). The extract (100 µL) was loaded on Silica gel 60 F$_{254}$ TLC plates (20 x 20 cm, Merck), developed by (A) formic acid/ethyl acetate/chloroform (10/40/50 v/v/v) or (B) formic acid/n-butanol/H$_2$O (5/95/50 v/v/v). Standard $^{14}$C-pyruvic acid was identified by autoradiography after exposure for 72 h (C). Each plate was divided into ten Rf's. Each Rf was scraped and applied on a microcolumn containing glass wool and eluted with formic acid (10 M). The eluate was further evaporated under N$_2$, dissolved in 500 µL distilled water, and the radioactivity was counted.

Figure 2. Determination of the molarity of formic acid required for the elution pyruvic and citric acids by column chromatography. Radioactive $^{14}$C-pyruvic or $^{14}$C-citric acids were added to the EtOH extracts of juice sacs. The EtOH extract was concentrated to 15 % of its volume, loaded on Dowex-1 column, eluted stepwise with 20 mL 0, 2, 4, 6, 8, 10 and 12 M formic acid. The eluates were evaporated to dryness, dissolved in 500 µL water and their radioactivity was counted.

$[^{12}]$C- and $[^{14}]$C-citric as well as $[^{12}]$C- and $[^{14}]$C-pyruvic acid markers were separated very effectively and were identified by autoradiography and by spraying TLC plates with Brom Cresol Green (R$_{f}$ 0.5–0.6, and R$_{f}$ 0.01–0.02, respectively; figure 5 A). When standard citric acid was added to the plant extract in the same system, it was accompanied by a strong tail of other organic acids (results not shown). Formic acid residues (10 µL) from the EtOH extract (see table I) were further chromatographed. The citric and pyruvic acid markers were identified according to their Rf's (figure 5 A). The radioactivity of the acid fractions was determined by a scintillation counter after scraping the spots of the acids on the TLC plate (figure 5 B).

For the preparative separation of the citric and pyruvic acids in the juice sac extracts, 120 µL radio-

pyruvic acid peak (figure 4) as shown in the HPLC chromatogram. It was, therefore, necessary to purify the extract by TLC prior to the HPLC analysis.

Figure 3. HPLC separation of selected organic acid standards. For details see Methods section 4.10.

Figure 4. HPLC separation of labeled organic acids originating from juice sacs incubated with either labeled $[^{14}]$C-sucrose (A) or $[^{14}]$C-fructose (B), compared with standard labeled $[^{14}]$C-pyruvic (C) and $[^{14}]$C-citric acids (D).
active residues (table 1) were chromatographed on TLC plates prior to further HPLC purification. Spots with the Rf values of citric and of pyruvic acids, as well as adjacent Rf's zones, were scraped yielding six strips altogether (figure 5 A). Each strip was loaded into a microcolumn and the acids were eluted with 15 mL 10 M formic acid. Each fraction was later evaporated under N2 and the residue was then dissolved in 500 μL distilled water. Radioactivity was determined in 50-μL samples. The first two strips contained most of the radioactivity and were identified according to the Rf values of citric and pyruvic acid markers. A much smaller amount of radioactivity was found in strip 4, which was identified as pyruvic acid (figure 5 B). Another 20 μL from the 500-μL aliquots of each strip extract (numbers 1–6), were injected into the HPLC column. Based on the Rf values of their standards (for citric acid, Rf = 15.3, tube 8; for pyruvic acid, Rf = 17.6, tube 9), citric acid was detected as expected in both the first and second strips (figure 6 A, B). This was confirmed by the radioactivity found in the same tubes (figure 7). Pyruvic acid was detected in the fourth strip (figure 6 C). Peaks at Rf values of 9.0–9.60 and 24.6–24.8 probably represent impurities originating from the extraction media since they appear consistently in every tested sample (figure 6 A–C).

The identity of citric acid was further established by a gas chromatography (GC) using the effluent from tube 8 obtained from strip 2 of the TLC chromatogram. After drying and methylation, the product was dissolved in chloroform and 1 μL was injected and analyzed. Citric acid was identified at Rf 12.35 (figure 8 C). This Rf value was identical to that of an authentic standard (figure 8 A, B) and was verified by spiking authentic citric acid in a purified extract sample from tube 8 (figure 8, compare peaks at Rf 12.36 in C and Rf 12.43 in D). Peaks at Rf values of 10.15, 14.00 and 16.05 (figure 8 C, D) are probably impuri-

Figure 7. HPLC separation of organic acids from strip 2 in figure 6. Juice sacs were incubated with $[^{13}C]$-sucrose (A) or $[^{13}C]$-fructose (B) for 16 h. Other details as described in the legend of figure 6.

Figure 8. GC separation of citric acid from juice sacs. A. Organic acid standards; B, citric acid standard; C, juice sac extracted with EtOH after incubation for 16 h with $[^{13}C]$-fructose, purified by TLC (strip 2, figure 5) and HPLC (test tube No. 8, figure 6); D, spiking of authentic citric acid to C.

3. DISCUSSION

Under most storage conditions there is an increase in the total amount of soluble sugars in citrus juice cells with a concomitant decrease in organic acids [7, 13, 18, 22]. This observation plus the fact that citric acid has been shown to serve as substrate for respiration [20], as well as the reported lack of hexokinase in the soluble fraction of juice cell proplasts [14], led to the belief that the substrates for respiration in harvested citrus fruits are mainly organic acids. The apparent inability of these fruits to use simple sugars is quite surprising, however, since an increase in the glycolytic utilization of sugars is common in tissues subjected to conditions of low oxygen or chilling stress [6, 10]. In mature citrus fruits, an increase in the EtOH and acetaldehyde contents is an indication of increased anaerobic respiration [11, 21]. In plant cells, the reduction in cytosolic ATP as a result of low oxygen or chilling stress is partially circumvented by the increase in PFP, an enzyme whose activity is ultimately dependent on hexose phosphorylation. A reduction in the energy status of mature and stored citrus fruits (a decline in their ATP content) has been well-documented [4].
Table II. Distribution of hexokinases in subcellular fractions obtained from grapefruit juice sacs by differential centrifugation. Values are means ± SE of twenty experiments.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glucokinase activity (nkat·g⁻¹ FW)</th>
<th>Fructokinase activity (nkat·g⁻¹ FW)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(nkat·mg⁻¹ prot)</td>
<td></td>
</tr>
<tr>
<td>10,000 x g pellet</td>
<td>101.9 ± 5.7</td>
<td>1,212.4 ± 47.9</td>
</tr>
<tr>
<td>100,000 x g pellet</td>
<td>29.6 ± 2.2</td>
<td>408.7 ± 26.9</td>
</tr>
<tr>
<td></td>
<td>86.3 ± 5.0</td>
<td>1,016.9 ± 49.8</td>
</tr>
<tr>
<td></td>
<td>27.4 ± 1.8</td>
<td>379.8 ± 23.2</td>
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In this report, we present convincing evidence indicating that citrus juice cells are capable of utilizing stored sucrose at maturity and after harvest as a source of energy via the TCA cycle. This conclusion is based on two lines of evidence. First, feeding experiments demonstrated the utilization of glucose, fructose and sucrose by grapefruit juice cells (table I; figures 1, 4, 7, 8 C, D). The formation of $^{14}$CO$_2$, $^{14}$C-pyruvate and $^{14}$C-citrate from the supplied $^{14}$C-sucrose or $^{14}$C-fructose strongly supports this contention. The possibility, however, that at least part of the $^{14}$CO$_2$ originating from either $^{14}$C-glucose, $^{14}$C-fructose or $^{14}$C-sucrose substrates was derived from the pentose phosphate pathway [23] cannot be ruled out. Because of the interconversion of fructose-6-P to glucose-6-P in the cytosol, the metabolism after feeding with either glucose or fructose includes both glycolysis and the pentose phosphate pathway. We therefore considered that fructose would be as relevant as glucose for feeding experiments. When using $^{14}$C-U-fructose-sucrose, the $^{14}$CO$_2$ originates only from fructose. The amount of $^{14}$CO$_2$ evolution as a percentage of the total uptake radioactivity was, however, rather small during the experimental period. This was also true for the amount of radioactivity left in the residue at the end of the washing procedure (table I). This may reflect the low respiratory rates of the fully mature citrus fruit [1]. The low uptake of sugars at a mature stage of fruit development may be a result of the reduced amounts of sucrose synthase and invertase [19]. These enzymes, however, might not be a significant factor in sucrose hydrolysis in the juice sac cells; the main factor might be the hydrolysis of vacuolar acids, causing only a weak component of sink strength [12]. Another reason for the low sugar uptake may be related to the experimental system used. In the present study, the juice sacs were cut on both ends to form 3-mm sections, allowing only some broken cells to be exposed to the medium. If the transport of solutes in the cells of juice sacs is symplastic, as suggested by García-Luis et al. [17], for young fruits still attached to the albedo (the white portion of the peel) and the cells of the mature fruit are characterized by low sink strength, the uptake of the sugars is expected to be much lower in the mature juice sac cells than in the juice sac cells of young fruit [17].

The second line of evidence that mature juice cells can utilize sucrose comes from the demonstration of marked hexokinase activity (table II). Hexokinase activity would be necessary for the phosphorylation of hexoses prior to their entry into either the glycolytic pathway or the pentose phosphate pathway. Our results (table II) can explain the reported failure to measure this enzyme in fractions of protoplast extracts [14]. Considerable care was taken in this study to verify the identity of the products of sucrose and fructose utilization by juice cells. Although they differed in amounts and proportions, the radiolabeled products obtained with each of these sugars as substrate were similar (table I). $^{14}$C-Fructose was incorporated more efficiently into $^{14}$C-pyruvate and $^{14}$C-citrate than $^{14}$C-sucrose. This is probably because fructose was directly accessible to hexokinase activity as it entered the cytosol from the bathing solution. In contrast, $^{14}$C-sucrose would also have to enter the vacuole prior to hydrolysis in order to be utilized. Once hydrolyzed in the vacuole by the low vacuolar pH [12], the glucose and fructose products become diluted in the large internal pool. $^{14}$C-Fructose enters the glycolytic pathway directly via the cytosol, avoiding dilution in the internal vacuolar pool.

The supply of carbon skeletons for energy needs and for maintenance of physiological and biochemical activities in harvested citrus fruits is likely to be a highly regulated process. In this report, we demonstrate that, despite previous indication to the contrary, soluble sugars can serve as glycolytic substrates. This may explain the increase in sugar utilization observed by Roe et al. [21] with grapefruit under imposed anaerobic conditions. Utilization of sugars and acids can both occur in a parallel manner, with the rates and conditions of respiration determining the ratios of substrate utilization. Respiratory rates in mature citrus fruits are low [1] and consequently, the utilization of sugars can be expected to reflect these low rates of respiration.

4. METHODS

4.1. Plant material

Mature fruits were obtained from 16-year-old grapefruit trees (Citrus paradisi Macf cv. Marsh seedless) grafted on Troyer citrange (Citrus sinensis [L.] Osbeck × Poncirus trifoliata) rootstocks grown at the experimental farm at the Faculty of Agriculture, the Hebrew University of Jerusalem, Rehovot. Five mature fruits were picked separately from each of five trees and brought immediately to the laboratory. The fruits were peeled and the endocarp, containing the juice sacs, was used for the experiments. The membranes of each segment were carefully removed and six segments were randomly selected from each fruit. Sections (2–3 mm thick, 8–10 mm wide) from each were used as a model representing the juice sacs. Each replicate consisted of 18–24 sections (a total of 20–22 g) from segments of five fruits. Six replicates were used for each treatment.

4.2. Pre-incubation

Each of the six aforestated replicates was divided into six 25-mL Erlenmeyer flasks which included 5 mL MES buffer (250 mM, pH 5.6) containing 50 mM CaCl₂ and 150 mM mannitol. Preincubation with the buffer was required in order to wash the cut surface of the juice sac sections (about pH 2.0), thus preventing the acidification of the incubation media (pH 5.6). The flasks were gently agitated for 5 min. The buffer was subsequently removed by mild vacuum, replaced with 5 mL of the same buffer and gently agitated again for 5 min.

4.3. Incubation

The buffer was removed after the second agitation and replaced by 5 mL of the same buffer containing ampicillin (20 µg·mL⁻¹), neomycin (20 µg·mL⁻¹), micostatine (20 µg·mL⁻¹), 18.3 kBq of either [¹⁴C]-U-glucose (10.73 MBq·mmole⁻¹), or [¹³C]-U-fructose (10.73 MBq·mmole⁻¹) or [¹⁴C]-U-fructose-sucrose (11.38 MBq·mmole⁻¹) or [¹³C]-U-sucrose (22.76 MBq·mmole⁻¹), and 0.2 mM of the corresponding [¹²C]-sugar as a carrier. Each flask was sealed with a serum stopper, which contained a sealed needle with three Whatman No.1 filter paper discs (9 mm in diameter). Soluene-350 (25 µL) was added to each disc as a [¹⁴C]CO₂ trap. The sealed flasks were incubated in a waterbath shaker for 6–16 h at 25 °C in the dark.

4.4. Measurements of [¹⁴C]CO₂ evolution

At the end of the incubation period, the serum stoppers were removed. The incubation buffer was transferred into a new 25-mL flask and resealed with the same serum stopper, containing the sealed needle with the three filter paper discs (leaving the juice sacs in the flask). The juice sacs were further kept on ice during analysis of sugar uptake (described in the following section). The stopper was removed from each needle, 2 mL 6 M H₂SO₄ were injected through the attached needle into the incubation solution, and the needle was sealed immediately after. The flasks were incubated 30 min in a waterbath shaker at 25 °C in the dark. Following this second incubation, the filter paper discs from the same six flasks (equal to one replicate) were combined in a 20-mL scintillation vial containing 10 mL scintillation solution. The vials were shaken for at least 3 h before [¹⁴C]CO₂ determination. The solution media from the six flasks (from the same treatment) were combined and examined for total radioactivity.

4.5. Determination of sugar uptake into the juice sacs

The juice sacs in six Erlenmeyer flasks, representing one replicate (with six replicates for each treatment), were combined and washed three times (each time 15–30 s) with 10 mL pre-incubation buffer. The washed juice sacs of the six replicates were divided into two groups and further extracted with a mortar and pestle either in 85 % EtOH or in 5 % TCA. Care was taken to keep the washing fluid and the juice sacs at 4–6 °C.

4.6. Ethanol extraction

Washed juice sacs were extracted in 60–80 mL 100 % EtOH. The mortar and pestle were rinsed with 20 mL 100 % EtOH which was combined with the extraction solution. The combined solutions were incubated in 250-mL flasks for 30 min in an ice bath and shaken every 5 min for a few seconds. The solutions were then filtered through Miracloth and the filtrates centrifuged (10 min, 10 000 x g, 4 °C). Aliquots (0.5 mL) of the supernatant were transferred into scintillation vials for the determination of radioactivity uptake by EtOH extraction.

4.7. Trichloroacetic acid (TCA) extraction

Washed juice sacs were extracted with 1 mL 100 % TCA. The mortar and pestle were rinsed three times with 20 mL 5 % TCA. The combined extracts were incubated in 250-mL flasks and further treated as described above for the EtOH extraction. Aliquots (0.5 mL) of the supernatant were transferred into
scintillation vials for the determination of radioactivity uptake by TCA extraction.

4.8. Preparation for TLC, HPLC and GC analyses

The EtOH extract (100 mL) was evaporated and the remaining water phase was passed through a Dowex-1 column (1.5 x 5.0 cm) preconditioned with 20 mL 15 M formic acid and equilibrated with 100 mL distilled water to pH 5.0. Organic acids were eluted from the column with 20 mL 4 M formic acid. The formic acid eluate was evaporated to dryness in a rotavapor and the residue was dissolved in 500 µL double distilled water. The TCA extract (60-65 mL) was adjusted to pH 2.5 and extracted with 3 x 40 mL saturated ethyl acetate. The ethyl acetate fraction was evaporated to dryness in a rotavapor and its residue was dissolved in 500 µL double distilled water.

4.9. Identification of organic acids by TLC

TLC was performed using pre-coated Silica gel 60 F₁₅₄ plates (250 µm, Merck). Standard pyruvic and citric acids were used as references. Identification of the organic acids was based on their Rᵥ values in three solvent systems: (A) formic acid/ethanol chloroform (10/40/50 v/v/v); or (B) formic acid/n-butanol/H₂O (5/95/50 v/v/v) as developing systems for TCA extracts; and (C) formic acid/amyl formate/chloroform (15/70/15 v/v/v) as a developing system for the EtOH extracts. After development, each TLC plate was dried for 24 h. Identification of the organic acids was based on four methods: UV 254 nm lamp; autoradiography for 24-72 h with a Fugi Imaging Plate (20 x 40 cm) type BAS-III; development by 2% Brom Cresol Green in EtOH; and analysis of the radioactivity of the organic acid fractions by scintillation spectroscopy. The spots corresponding to the acids according to their Rᵥ value were scraped from the TLC plate, and the powder was placed in a vial to which 3.5 mL scintillation solution was added.

4.10. Identification of organic acids by HPLC

Before injection into the HPLC column, samples of the authentic standard and the residue of the formic acid eluates were filtered through a microfiber filter (0.45 µm). A HPLC 'A' Model SP 8000 B high-pressure liquid chromatograph with an integrated oven compartment and data system was used in combination with a Model R 401 refractive index detector at 254 nm. Samples were injected using a sample loop equipped with a 20-µL loop. An interaction ion-300 strong cation-exchange resin column (300 x 7.8 mm I.D.), fitted with an ion-exclusion MicroGuard refill cartridge (Bio-Rad Labs) was used. The pump pressure was 525 PSI. The temperature of the column was 75 °C. The eluant was 0.004 25 M sulfuric acid, prepared by dilution of 0.05 M analytical reagent grade sulfuric acid and degassed under vacuum for 30 min. During chromatography, it was degassed with helium. The authentic standards were obtained from Sigma. Each sample was chromatographed for 30 min (0.4 mL-min⁻¹) and fractions were collected by a fraction collector (0.8 mL-tube⁻¹). Radioactivity in test tubes was tested by adding 3.5 mL scintillation solution. In other chromatographic samples, the test tubes were dried and methylated with BF₃ prior to GC analysis.

4.11. Identification of organic acids by GC

Samples (1 µL) were injected into a Varian 3300 gas chromatograph equipped with a megabore DB-17 column (A&W, USA) at 50 °C (3 min), reaching 200 °C (20 °C-min⁻¹) and 200 °C hold for 5 min (total 17 min). Organic acids were detected using a flame ionization detector at 220 °C and injection at 200 °C. The carrier was N₂ (10 mL-min⁻¹) and Make-up N₂ (20 mL-min⁻¹).

4.12. Tissue preparation for hexokinase assay

One year earlier, hexokinase (EC 2.7.1.1) activity was determined in grapefruits collected from the same trees as in these experiments. Grapefruit juice sacs (10 g) were homogenized for 1 min in a Potter-Elvenjem containing 15 mL cold buffer solution consisting of 750 mM Tris-HCl (pH 8.0), 500 mM sucrose, 0.6 % PVP-40, 1 µM DTT and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged for 10 min at 1 000 x g to remove cell debris and cell nuclei. The pellet was discarded and the supernatant centrifuged for 20 min at 10 000 x g to collect the mitochondrial fraction. The pellet was collected and the supernatant centrifuged at 100 000 x g for 60 min to collect the microsomal fraction. Both pellets were resuspended in 0.5 mL 100 mM Tris-HCl (pH 8.0) and 1 mM DTT. Both samples were stored at ~80 °C until further use. Hexokinase activity was measured following the increase in absorbance at 340 nm in the presence of 100 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.5 mM NADP⁺, 3 mM ATP, 1 unit glucose-6-P dehydrogenase (free from glucuronate dehydrogenase) and 10 mM hexose (glucose or fructose for glucokinase (EC 2.7.1.2) or fructokinase (EC 2.7.1.4),

respectively). When hexokinase was assayed in the presence of fructose, 1 unit phosphohexoisomerase (EC 5.3.1.9) was added.

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