Compartmentation of SoluteS and Water in Developing Sugarcane Stalk Tissue

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ABSTRACT

Previous studies have suggested that the apoplast solution of sugarcane stalk tissue contains high concentrations of sucrose, but the accuracy of these studies has been questioned because sucrose leakage from damaged cells may have influenced the results. In this study, the solute potential of the apoplast and symplast of the second (immature), tenth, twentieth, thirtieth, and fortieth internodes of field-grown sugarcane (Saccharum spp. hybrid) stalk tissue was determined by two independent methods. Solute potential of the apoplast was measured either directly by osmometry from solution collected by centrifugation, or inferred from the initial water potential of fully hydrated tissue determined by thermocouple psychrometry before the tissue was progressively dehydrated for generation of water potential isotherms. Both methods produced nearly identical values ranging from −0.6 to −1.8 megapascals for immature and mature tissue, respectively. The solute potential of the symplast determined by either method ranged from −1.0 to approximately −2.2 megapascals for immature and mature internodes, respectively. Solute quantitation by HPLC agreed with concentrations inferred from osmometry. Washing thirtieth internode tissue in deionized water increased pressure potential from 0.29 to 1.96 megapascals. The apoplast of mature sugarcane stalk tissue is a significant storage compartment for sucrose containing as much as 25% of the total tissue water volume and as much as 21% of the stored sucrose.

MATERIALS AND METHODS

Plant Material.

Sugarcane (Saccharum spp. hybrid, cv H65-7052) plants were field-grown in a silty-clay loam (Typic Torrox) soil near Waipahu, Oahu, HI. Well-watered 1.5- to 2-year-old plants were excised prior to sunrise when guttation water was present on leaf margins, indicating that the plants were fully hydrated. After excision, plants were immediately sealed in plastic bags and transported to the laboratory. Tissue samples were collected and analyzed on the basis of internode number. The reference point for determining internode number was the TVD leaf (12); the youngest leaf whose blade is fully exposed and not enrolled in the sheaths of older leaves. The first internode was the node below the TVD leaf sheath attachment, and additional internodes were numbered consecutively down the stalk. A 2-year-old sugarcane stalk grown under the conditions described above typically contained approximately 50 internodes. In this study, internodes 2, 10, 20, 30, and 40 were analyzed.

Internode tissue sections were prepared for analysis inside a humidified chamber to reduce water loss. The stalk was cut in half at least 1 cm from the node and the epidermis and any pith tissue was removed with a knife, because the fiber content is higher and sucrose concentrations lower in these tissues (3, 6).

Aploplast Solute Potential

Determinations of \( \psi_u \) were obtained by two independent methods. In the first method, tissue samples approximately

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3 Abbreviations used: \( \psi \), water potential; \( \psi_s \), solute potential; \( \psi_u \), apoplast solute potential; \( \psi_b \), bulk tissue solute potential; \( \psi_s \), symplast solute potential; \( \psi_p \), pressure potential; RWC, relative water content; RN, relative apoplast water fraction; FWT, fresh weight; DWT, dry weight; TVD, top visible dewlap; WC, water content; RCF, relative centrifugal force.

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4.5 x 1 x 1 cm were placed in Centrex microfilter centrifuge tubes (Schleicher and Schuell) equipped with a 0.8 μm cellulose acetate filter and a 5.0 mL receiver tube. Tubes were centrifuged for 10 min at 30g then for an additional 10 min at 480g. The solution from the first centrifugation was discarded because it contained symplast contamination from cut surfaces and damaged cells (Fig. 1). The second centrifugation yielded at least 40 to 70 μL of liquid that was analyzed as apoplasm solution. The osmolality of the apoplasm solution was measured with a vapor pressure osmometer (Wescor 5100B) and the remaining sample was stored at −80°C for further analyses. Osmolality was converted to solute potential (at 25°C) by multiplying the value in Os·kg−1 by 2.48 MPa·Os−1·kg.

The second method involved measuring the internode tissue ψ psychrometrically. Tissue segments approximately 3 x 1 x 1 cm were sealed in psychrometer (Merrill 83) chambers (Merrill 81-500), and measured with a microvoltmeter (Merrill 85) in the psychrometric mode after equilibration for 3 h at constant temperature. The ψ values obtained were taken as measurements of ψsa since both parameters are equivalent in fully hydrated tissue. Psychrometers were calibrated biweekly with salt solutions of known ψs in the range of samples to be tested.

**Symplast and Bulk Tissue ψs**

The ψs was measured by three methods. In the first, the apoplasm solution was removed from the tissue by centrifugation. The tissue was then frozen and thawed, crushed (>160 MPa, Hydraulic Press, Carver, Inc.), and the ψ of the expressed solution was measured by osmometry. In the second method, the apoplasm solution was removed by centrifugation, the tissue was frozen and thawed, and the ψ of each piece of tissue was measured by psychrometry. In the third method, ψs at full turgor was determined from ψ isotherms by plotting 8 to 10 values of 1/ψ, measured psychrometrically, as a function of tissue RWC (water content/water content at full hydration) and extrapolating the linear portion of the plot to the ordinate (16). The initial ψ measurements were made on freshly harvested, fully hydrated tissue. Subsequent measurements were made after samples had dried in the open at room temperature for 20 to 30 min. The ψs was measured by osmometry from solution expressed from frozen and thawed, crushed tissue.

**Pressure Potential**

Two methods were used to determine ψs. Following centrifugation, ψs was obtained from the difference between ψsa and ψsb measured by osmometry. Turgor was also determined from ψ isotherms by subtracting ψsa, extrapolated to the ordinate, from ψ measured psychrometrically.

**Relative Apoplasm Water Volume**

The Rsa of stalk tissue was determined by two methods. In the first, the linear portion of the ψ isotherm was extrapolated to the abscissa (RWC) at 1/ψ = 0 to obtain an estimate of the symplasmic water fraction (16). This value was subtracted from 1.0 to obtain Rsa. By the second method, ψsa was assumed to be a weighted average of ψs and ψsb according to the relationship:

\[
\psi_{sa} = \frac{\psi_{sb} - \psi_{ss}}{\psi_{sa} - \psi_{ss}}
\]

where ψsa is the ψs of the apoplasmic solution extracted by centrifugation. Solving for Rsa yields:

\[
R_{sa} = \frac{\psi_{sb} - \psi_{ss}}{\psi_{sa} - \psi_{ss}}
\]

**Solute Identification**

Apoplasm and symplasm solutions were analyzed for sugars and organic acids by HPLC. Sucrose, fructose, and glucose were separated using a Shodex SC1011 column (Alltech Assoc. Inc.) and detected with a differential refractometer, while organic acids were separated using an Ion-300 column (Interaction Chemicals Inc.) and a UV detector at 215 nm. Potassium concentration and pH were determined using microelectrodes (Microelectrodes, Inc.).

**Water Content, Percent Soluble, and Insoluble Solids**

Internode tissue was weighed, heated at 70°C for 24 h and reweighed to determine FWT, DWT, and WC (FWT basis). The percentage of soluble solids was calculated from the concentration data in Table II, the FWT, and the WC. The weight of the soluble solids was subtracted from the tissue DWT and divided by the FWT to determine the % insoluble solids.

**RESULTS**

**Solute Potential as a Function of Relative Centrifugal Force**

Tissue from the 10th internode was consecutively centrifuged at 30, 121, 480, 3,020, 6,780, and 12,100g, and the ψs.
of each fraction was measured to test for evidence of symplast contamination of the apoplastic solution during centrifugation (Fig. 1). The $\psi_i$ increased from $-1.74$ MPa at 30g to the highest value of $-1.48$ MPa at 3,020g then declined to $-1.74$ MPa at 12,100g. After the final centrifugation at 12,100g, the tissue was frozen, thawed, and crushed, and the $\psi_i$ of the expressed solution was $-2.03$ MPa. Submerging internode tissue briefly in water and blotting to remove the excess, raised $\psi_i$ values by roughly 0.4 MPa (data not shown). However, we felt this procedure underestimated apoplastic solute potentials because of losses due to leaching. Thus, samples were routinely centrifuged at 30g, to remove contamination from cut surfaces. A second centrifugation was then performed at 480g, and the fraction collected was analyzed as apoplast solution (Fig. 1). The solutions collected during the centrifugations to remove cut surface contaminants and the apoplast solution accounted for 1.5 and 4.3% of the total stalk tissue solution, respectively. The remaining 94.2% of the solution remaining in the stalk tissue following centrifugation was considered to be largely symplast.

**Components of Symplast and Apoplast $\Psi$**

Representative $\Psi$ isotherms, for the 2nd and 30th internodes are shown in Figure 2, A and B. Average values of $\Psi_{sy}$, $\Psi_a$, and $\Psi_s$ obtained from $\Psi$ isotherms and by the centrifugation of tissue pieces from internodes 2, 10, 20, 30, 40 are shown in Figure 3. With the exception of the 10th internode, $\psi_p$ values determined from $\Psi$ isotherms were almost constant ranging from 0.30 to 0.42 MPa (Fig. 3A). Pressure potential calculated by subtracting $\psi_i$ from $\Psi$ (determined by centrifugation and osmometry) increased from 0.18 MPa at the 2nd internode to 0.40 MPa at the tenth internode and remained essentially constant thereafter (Fig. 3A).

Values of $\psi_a$ inferred from the $\Psi$ of fully hydrated tissue pieces measured by psychrometry were in good agreement with those obtained for apoplast solution extracted by centrifugation and measured by osmometry (Fig. 3B). Apoplast $\psi_a$ decreased sharply from approximately $-0.75$ MPa in the 2nd internode to a minimum of $-1.7$ to $-2.0$ MPa in internodes 20 thru 40 (Fig. 3B).

The $\psi_s$ was very similar whether measured from crushed tissue following centrifugation or by extrapolation from $\Psi$ isotherms, dropping from $-1.0$ MPa at the 2nd internode to a minimum of approximately $-2.2$ MPa from internodes 20 to 40 (Fig. 3C).

Tissue samples from the 30th internode were also washed in water for 1 h prior to $\Psi$ isotherm determination. Washing increased $\psi_p$ from 0.29 to 1.96 MPa, decreased $\psi_a$ from $-1.94$ to $-0.27$, decreased $R_a$ from 25.1% to 18.5%, and increased WC from 73.8% to 88.6% (cf. Fig. 2, B and C; Table 1).

**Relative Apoplastic Volume**

The $R_a$ was determined by extrapolation from $\Psi$ isotherms or from Equation 2. The $R_a$ increased with internode age and ranged from 4.1 to 20.2% and 12.5 to 29.2% for the centrifugation and $\Psi$ isotherm methods, respectively (Fig. 4).
Table I. Water Relations of Washed and Untreated Sugarcane Stalk Tissue

Water potential isotherms were used to analyze tissue pieces from the 30th internode following a 1 h wash treatment in deionized water, or directly as obtained from newly harvested, fully hydrated plants. The average for all unwashed tissue is shown as well as an untreated control determined from subsamples measured simultaneously with the washed tissue. Averages were taken from 10 replicates (unwashed) and 4 replicates (control and washed).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>( \psi_{so} ) (MPa)</th>
<th>( % \text{ of total vol} )</th>
<th>( % \text{ of FWT} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of all</td>
<td>-1.94 ± 0.12</td>
<td>27.3 ± 4.1</td>
<td>73 ± 2.1</td>
</tr>
<tr>
<td>± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unwashed</td>
<td>-1.64 ± 0.20</td>
<td>25.1 ± 3.0</td>
<td>73.8 ± 1.9</td>
</tr>
<tr>
<td>± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Washed</td>
<td>-0.27 ± 0.07</td>
<td>18.5 ± 2.0</td>
<td>88.6 ± 3.4</td>
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<td>± SE</td>
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Identity of Symplastic and Apoplastic Solutes

The solute concentrations determined by HPLC and the \( K^+ \) electrode accounted for 100% of the osmolality in the 2nd internode, and about 80% in older internodes. Organic acids, fructose, glucose, sucrose, and \( K^+ \) each accounted for at least 11% of the total osmolality in each compartment at the 2nd internode, while in mature internodes sucrose was the predominant solute in both compartments (Table II). Sucrose content increased from the 2nd internode to the 30th internode and decreased slightly at the 40th internode. Fructose and glucose levels decreased rapidly from the 2nd internode until levels at the 20th internode were barely detectable and essentially nil, respectively (Table II). Potassium concentrations were essentially the same in both compartments, decreasing slightly after the 2nd internode and then remaining constant in mature tissue.

Organic acids accounted for over 30% of the measured osmolality in the 2nd internode with concentrations of greater than 100 mM present in both compartments (Table II). The concentration of organic acids declined with maturity, and at the 10th and 40th internodes concentrations were less than one-third those at the 2nd internode (Table II). In the 2nd internode, succinate and aconitate were the only detectable organic acids in the apoplast with concentrations of 76 and 44 mM, respectively (Table III). In the symplast, similar quantities of succinate and aconitate were present in addition to measurable quantities of citrate, malate, and oxalate (Table III). At the 40th internode, succinate was not detected in either compartment, and aconitate accounted for at least 75% of the organic acids.

The pH of the symplast solution was 5.06 at the second internode, increasing to a plateau of 5.42 from internodes 10 to 30, and then decreasing to 5.21 at the 40th internode (not shown). The pH of the apoplast solution was 5.34 at the 2nd internode, increasing gradually to a maximum of 5.60 at the 30th internode, and then decreasing to 5.31 at the 40th internode.

Water Content, Percentage of Soluble and Insoluble Solids

Tissue WC declined from 84% at the 2nd internode to a minimum of 77% in mature internodes (Fig. 5A). Both the soluble and insoluble solids increased after the 2nd internode to constant values of 19 and 9%, respectively, in mature tissue (Fig. 5A). The percentage of total sucrose in the apoplast increased from 7.5% in the 2nd internode to 21.6% in the 40th internode (Fig. 5B).

DISCUSSION

In a previous study, Hawker (9) measured sucrose concentrations as high as 20% in the apoplast of sugarcane stalk tissue. This value was obtained by comparing the sucrose concentration of washed and unwashed 1 mm thick tissue discs. This technique has been criticized, because the mechanical damage suffered during preparation and washing may cause the tissue to behave differently than in an intact plant (5). In the present study, the \( \psi \), of the apoplast solution was either (a) determined psychrometrically from fully hydrated stalk tissue or (b) determined by osmometry following isolation by centrifugation at low RCF. Because the \( \psi \) of bulk tissue is determined by the apoplast solution, the psychrometric measurement of tissue \( \psi \) is also a measurement of \( \psi_{so} \). However, consideration must be given to several types of systematic errors that may adversely influence psychrometric measurements. If barriers to water vapor movement were present in the sample or insufficient equilibration time were allowed, the thermocouple may have only equilibrated with the outer cell layers of the tissue which were predominantly composed of damaged cells. With the exception of the rind tissue, which was removed during sample preparation, there are no barriers to water vapor movement associated with internode tissue (2). Water potential values were constant.
after a 3 h equilibration period and remained constant for up to 12 h indicating that equilibration with the entire sample had been achieved (data not shown). During the preparation of tissue for measurement by psychrometry, solutes released from cut surfaces may be reabsorbed by surrounding cells causing \( \psi_s \) to decrease and \( \psi_r \) to increase resulting in an overestimate of \( \psi_w \). This phenomenon can be observed as a constant \( \Psi \) that persists on \( \Psi \) isotherms during successive measurements even though the RWC is reduced below 100%.

In this study, relatively large \( 3 \times 1 \times 1 \) cm tissue slices were used, and reductions in \( \Psi \) accompanied reductions in RWC, resulting in continuous \( \Psi \) isotherms (Fig. 2). The lack of any detectable solute reabsorption may be due to the fact that cut cells comprise a smaller percentage of the total tissue in large slices than in small slices. Therefore, the effect of cut surfaces on the bulk tissue \( \Psi \) measurements is probably less in large slices used in this experiment than in small tissue slices used by previous investigators (8, 9).

The centrifugation technique used to isolate the apoplastic solution can also produce erroneous results if its limitations are not understood. Terry and Bonner (14) used centrifugation to isolate cell wall polysaccharides from pea stems, and found that leakage from the symplast began at 3000g. In the current study, the apoplastic solution was collected at 480g, well below the force reported to induce leakage. Analyses of immature internode tissues in other species of *Saccharum* that accumulate sugars have produced \( \psi_w \) values greater than \(-0.5 \) MPa, indicating that the high apoplastic solute concentrations were not due to systemic errors in the centrifugation technique (our unpublished results). Isolation of the apoplastic solution by previous investigators (8, 9), has required vacuum infiltration to obtain sufficient quantities of solution for analysis (4, 14). While the hydrated sugarcane stalk yielded sufficient solution for repeated analyses without the addition of water. Contamination from the phloem during centrifugation would not be expected to constitute a large error, since the phloem has been estimated to comprise less than 0.5% of the volume in mature stalk tissue (9).

In agreement with previous studies (7, 9), our results indi-

| Table II. Principal Solutes in the Apoplastic Solution Extracted from Sugarcane Stem Internodes by Centrifugation and in the Symplast Solution |
|-----------------------------|---------------------|---------------------|
| Solute                      | 2                   | 10                  |
|                             | Osmolarity (%) of   | Osmolarity (%) of   |
|                             | total               | total               |
| Symplast                    |                     |                     |
| Sucrose                     | 110 ± 26            | 26                  |
| Fructose                    | 55 ± 10             | 13                  |
| Glucose                     | 62 ± 12             | 15                  |
| K+                          | 71 ± 4              | 17                  |
| Organic acids               | 135                 | 32                  |
| Unidentified                | 0                   | 0                   |
| Total                       | 417 ± 28            | 792 ± 40            |
| Osmolality kg⁻¹             |                     |                     |
| 2                           |                     |                     |
| 10                          |                     |                     |
| 20                          |                     |                     |
| 30                          |                     |                     |
| 40                          |                     |                     |
| Apoplast                    |                     |                     |
| Sucrose                     | 102 ± 21            | 29                  |
| Fructose                    | 43 ± 7              | 12                  |
| Glucose                     | 40 ± 8              | 11                  |
| K+                          | 72 ± 4              | 20                  |
| Organic acids               | 120                 | 34                  |
| Unidentified                | 0                   | 0                   |
| Total                       | 352 ± 25            | 629 ± 21            |

* Indicates data not available.
icate that the apoplast of field-grown sugarcane contains a high concentration of osmotically active solutes. Since two independent methods produced similar estimates of $\psi_{ap}$ (Figs. 2B, 3B), it is unlikely that these values were artifactual. In addition, there was good agreement between the solute concentrations determined by osmometry and by HPLC. Compartmental analysis showed that the composition of solutes in the apoplast and symplast was quite similar throughout development, although there were qualitative differences in the organic acids (Tables II and III). Aconitate and succinate were present in both compartments, while other TCA cycle acids were largely restricted to the symplast particularly in young tissue, indicating that aconitate and succinate were either preferentially leaked from the symplast or transported via the apoplast during translocation. The pH in both compartments was less than 5.6 suggesting that the symplast was predominantly composed of vacuoles and that much of the organic acids may have been in the form of K$^+$ salts.

Further evidence of high apoplastic solute concentrations was provided by the increase in $\psi_r$ determined from $\Psi$ isotherms following a washing treatment (Table I). Replacing the apoplast solution with deionized water increased $\psi_r$ to values nearly equaling the absolute value of $\psi_{ap}$, while at the same time, $\psi_{ap}$ increased to nearly zero (Fig. 2; Table I). Infiltrating red beet tissue discs with water elevated $\psi_{ap}$, while high $\psi_r$-induced solute leakage from the symplast (15, 18). In this study, $\psi_{ap}$ and $\psi_r$ values were unchanged during the 3 to 5 h needed to measure the $\Psi$ following washing, indicating that no significant solute leakage occurred. Hawker showed that tissue discs incubated in water lost sucrose rapidly during the first 40 min, and little additional sucrose was lost from 40 to 180 min (9). In studies using a pressure probe, turgor in sugarcane stalk tissue cells increased dramatically and remained high following a 3-h wash and incubation treatment (PH Moore, DJ Cosgrove, unpublished results). Thus, in sugarcane, solute leakage from the symplast is evidently not a short-term response induced by high $\psi_r$.

Other evidence indicates that in situ, long-term leakage from the symplast may occur. The WC and percentage of soluble and insoluble solids each reach a plateau by the 20th internode (Fig. 5A). However, the $R_a$ and percent of total sucrose in the apoplast continue to increase (Fig. 4, 5B). The increase in $R_a$ may be due to secondary wall thickening in immature tissue. However, in mature tissue, the percentage of insoluble solids was constant indicating that secondary wall thickening was complete (Fig. 5A). From internodes 20 to 40, both $\psi_r$ and $\psi_{ap}$ determined by centrifugation declined slightly (Fig. 3, A and B). Since the percentage of soluble solids and WC remained constant, the loss of $\psi_r$ may be mediated by solute leakage from the symplast and the consequent redistribution of tissue water. Solute leakage along with secondary wall thickening in young tissue could explain the steady increase in $R_a$ observed throughout development even though WC was constant and solids accumulation had ceased (Figs. 4 and 5A). The $R_a$ of 30th internode tissue decreased following washing, indicating that solute accumulation in the apoplast may contribute to increases in $R_a$ without additional cell wall synthesis (Table I).

Previous reports have also indicated that the free space volume ($R_a$ + air space) in sugarcane stalks increases during development. Bielecki reported that the free space comprised 10 to 18% and 15 to 21% of the total volume in immature and mature sugarcane tissue, respectively (1). Oworu et al. (13) reported free space values ranging from 9.7 to 24.1% and 14.8 to 57.7% in immature and mature tissue, respectively. Glassiou and Gayler (8) reported values as high as 80% for mature stalk tissue, a likely overestimate, since only 0.5 mm thick tissue slices were used. Hawker, studying only one stage of development, reported $R_a$ values of 13.6 and 14.3%, which agrees with the average $R_a$ determined for internode 20 in this study (9; Fig. 4). Our results, along with information from previous investigators, indicate that the apoplast comprises a significant percentage of the volume in mature sugarcane stalk tissue (Fig. 4). The apoplast of sugarcane stalk tissue contains high concentrations of solutes, and in mature internodes the predominant solute is sucrose (Table II; 7, 9). The apoplast thus represents an important storage compartment, and in mature tissue may contain over 20% of the total sucrose.

The existence of high apoplastic solute concentrations in sugarcane stalk tissue challenges some accepted thinking about whole plant water relations. Vascular bundles in sugarcane stalks are separated from the storage tissue by only one or two layers of bundle sheath cells. Diffusion of solutes from the apoplast into the xylem would seem possible especially considering that the concentration gradient can be maintained for 2 or more years. If the xylem is not contaminated by apoplastic solutes, the $\Psi$ of fully hydrated sugarcane plants should approach 0.0 MPa. The $\Psi$ of nongrowing sugarcane leaf tissue has been reported to be approximately −0.25 MPa, the $\psi_r$ of guttation water to be approximately −0.03 MPa, and the xylem pressure to be zero (12). Yet the $\Psi$ of isolated stalk internode tissue is as low as −1.8 MPa (Fig. 3B). The mechanisms by which fully hydrated intact sugarcane plants are able to attain $\Psi$ values near 0 MPa despite the existence of high apoplastic solute concentrations in the stem will be the topic of a later report.

**Figure 5.** A, Changes in WC and percentage of soluble and insoluble solids during development; B, the percentage of total sucrose in the apoplast determined by multiplying the apoplastic sucrose concentration by the average of the two apoplastic volume estimates shown in Figure 4 and then dividing by the bulk tissue sucrose concentration.
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LITERATURE CITED

6. Fernandez AC, Benda GTA (1985) Distribution patterns of brix and fibre in the primary stalk of sugar cane. Sugar Cane No. 5: 8–13