Leaf Carbon Export and Nonstructural Carbohydrates in Relation to Diurnal Water Dynamics in Mature Oak Trees[1][OPEN]

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Trees typically experience large diurnal depressions in water potential, which may impede carbon export from leaves during the day because the xylem is the source of water for the phloem. As water potential becomes more negative, higher phloem osmotic concentrations are needed to draw water in from the xylem. Generating this high concentration of sugar in the phloem is particularly an issue for the ~50% of trees that exhibit passive loading. These ideas motivate the hypothesis that carbon export in woody plants occurs predominantly at night, with sugars that accumulate during the day assisting in mesophyll turgor maintenance or being converted to starch. To test this, diurnal and seasonal patterns of leaf nonstructural carbohydrates, photosynthesis, solute, and water potential were measured, and carbon export was estimated in leaves of five mature (>20 m tall) red oak (*Quercus rubra*) trees, a species characterized as a passive loader. Export occurred throughout the day at equal or higher rates than at night despite a decrease in water potential to −1.8 MPa at midday. Suc and starch accumulated over the course of the day, with Suc contributing ~50% of the 0.4 MPa diurnal osmotic adjustment. As a result of this diurnal osmotic adjustment, estimates of midday turgor were always >0.7 MPa. These findings illustrate the robustness of phloem functioning despite diurnal fluctuations in leaf water potential and the role of nonstructural carbohydrates in leaf turgor maintenance.

Leaves are tasked with meeting the carbon and energy demand of the rest of the plant. Because leaves have a limited capacity to store carbohydrates, over a 24-h period all of the newly fixed carbon must, on average, either be consumed locally in respiration or exported. What then determines the diurnal pattern of carbon export from leaves? One possibility is that export is maintained at a constant rate, where carbon stored as starch when photosynthetic rates are high is used to support export during the night. Alternatively, export could scale with assimilation rates and thus be highest at midday. An inverse pattern might also occur if diurnal declines in leaf water potential (Ψ) impede the build-up of turgor in the phloem when transpiration rates are high.

For herbaceous plants, export occurs predominantly during the light period (e.g. Fondy and Geiger, 1982; Hendrix and Huber, 1986; Cure et al., 1991; Leonardos et al., 2006). However, unlike herbaceous plants, woody plants typically experience large diurnal variation in leaf Ψ (Tyree and Ewers, 1991), and the resulting impact on phloem export from leaves is poorly understood. Four-year-old poplar (*Populus* spp.) trees are reported to have higher export rates during the day than at night (Davey et al., 2006), whereas one-year-old Eucalyptus (*Eucalyptus globulus*) trees that reach a minimum (midday) leaf Ψ of −1.4 MPa have greater export rates at night than during the day (Quick et al., 1992). Additionally, for mature Scots pine (*Pinus sylvestris*) trees, phloem transport rate, estimated based on osmotic

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potentials, decreases during the day (Paljakka et al., 2017). Here, we document carbon export patterns in mature (>20 m tall) red oak (*Quercus rubra*) trees, in the context of water relations, to shed light on how leaf water status affects phloem functioning.

According to the Münch mechanism of osmotically driven pressure flow, the osmotic potential of the phloem must be more negative than the Ψ of the xylem in order to draw water into the phloem by osmosis (Münch, 1930). In red oak, where midday Ψ can fall to values around −2 MPa (Cavender-Bares and Bazzaz, 2000; Rockwell et al., 2014), the solute concentration of the phloem must be large enough to develop positive pressure for flow to occur. Only a handful of reports of red oak phloem sap sugar concentrations or total osmolality exist. Two of these reports indicate that export is not possible at midday because concentrations are not high enough to generate positive pressure in sieve tubes, whereas the third report cites a concentration on the cusp of being able to produce phloem pressure (Münch, 1930; Hammel, 1968; Zimmerman and Milburn, 1975). Additionally, red oak has been characterized as a passive loader. In passive loaders, sugar (specifically Suc) concentrations in the cytoplasm of mesophyll cells must be higher than the concentration in the phloem in order for sugars to move by diffusion from the sites of production to the sieve element of the phloem (Rennie and Turgeon, 2009). This means that in addition to having to build up their Suc concentrations in the phloem at midday, passive loading species also have to accumulate Suc in their mesophyll cytoplasm.

Whether as a consequence of reduced export or to drive diffusion to the phloem, increased mesophyll Suc concentrations may help the leaf with another midday challenge: mesophyll turgor maintenance. A number of studies have explored diurnal sugar patterns in herbaceous plants, finding that sugars peak at various times during the day: in the morning (barley [*Hordeum vulgare*; Sicher et al., 1984]), at midday (cotton [*Gossypium hirsutum*; Mason and Maskell, 1928], many C4 species [Lunn and Hatch, 1995], and potato [*Solanum tuberosum*; Urbanczyk-Wochniak et al., 2005]), and afternoon (soybean [*Glycine max*; Upmeyer and Koller, 1973], lupines [*Lupinus spp.*; Sharkey and Pate, 1976], sugar beet [*Beta vulgaris* 'F58-554H1'; Rao et al., 1990], barley, and spinach (*Spinacia oleracea*; Riens et al., 1994]). For trees, we are aware of a handful of studies of diurnal foliar carbohydrate levels. Of these studies, two do not show a significant diurnal trend in red maple (*Acer rubrum*), red oak, paper birch (*Betula papyrifera*), or quaking aspen (*Populus tremuloides*; Collier et al., 1992), or in almond (*Prunus dulcis*; Tixier et al., 2018), and four do show a trend (poplar [*Populus deltoids*; Dickson, 1987], cork oak [*Quercus suber*; Faria et al., 1996], apple [*Malus domestica*; Klages et al., 2001], and orange [*Citrus sinensis*; Ribeiro et al., 2012]).

By measuring diurnal and seasonal changes in water and solute potential, nonstructural carbohydrates (NSCs), and net assimilation, as well as calculating phloem export in mature oak trees, we tested two hypotheses regarding carbon and water dynamics in red oak leaves: (1) carbon export occurs primarily at night due to relaxed (less negative) Ψ; and (2) sugar accumulation in mesophyll cells allows red oak leaves to maintain turgor despite declines in midday Ψ. Our study is motivated by the idea that quantifying patterns of export and nonstructural carbohydrates in the context of leaf water relations will provide insight into how carbon dynamics interact with phloem and mesophyll turgor in the face of diurnal and seasonal water stress.

RESULTS

Carbon Export

Carbon export, estimated using a mass balance approach that integrates measurements of net assimilation and NSCs, was greatest during the day for each of the four seasonal time points (Fig. 1), with the maximum on average occurring at midday. The rate at which carbon was estimated to be exported from the leaf during the day was typically lower than net assimilation (A_N; Fig. 2), and this mismatch of export and A_N resulted in accumulation of NSCs in the leaf over the course of the day (Fig. 3).

There was no evidence that midday water stress impeded phloem function. In August and September, midday leaf Ψ was ~0.6 MPa lower than in June (Fig. 2), yet export reached similar levels (Fig. 1). In addition, during the afternoon, export levels fell in accordance with reduced assimilation, rather than increasing with the relaxation of leaf Ψ. Of the three export models (constant, proportional to assimilation, and inverse to

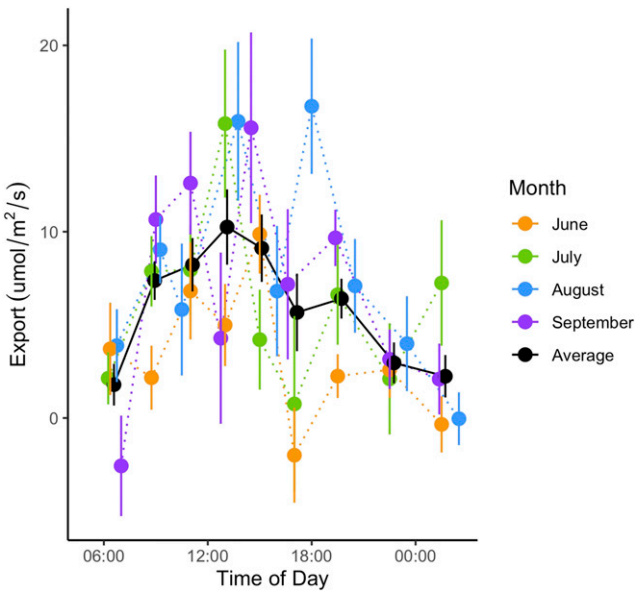

Figure 1. Diurnal patterns of carbon export for red oak leaves. Each monthly time point represents the average (± SE) of 15 leaves (three per tree). The average of all months (± SE) is shown in black.

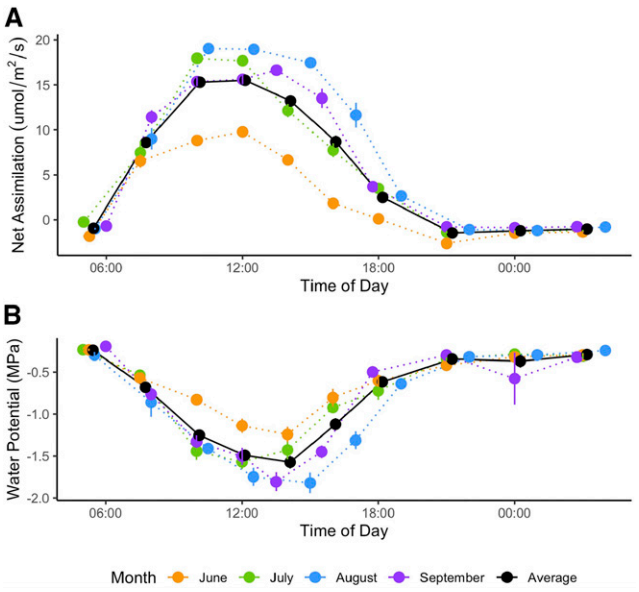

Figure 2. Diurnal patterns of net assimilation and leaf water potential for red oak leaves. For net assimilation (A), each monthly time point represents the average (± SE) of 20 leaves (four per tree), while for water potential (B) each monthly time point represents the average (± SE) of five leaves (one per tree). The average over all months (± SE) is shown in black.

water potential), the export data are most closely aligned with the proportional-to-assimilation model.

NSC Concentrations and Composition

Foliar sugars (specifically Suc, Glc, and Fru measured with an enzymatic assay) increased by an average of 16 mg g^{-1} dry weight (DW) during the day and returned to baseline values at night (Fig. 3). The main driver of the diurnal trend in total sugars was Suc; Fru and Glc remained relatively constant over the day (Fig. 4). Additionally, the baseline (minimum) amount of Suc in the leaf typically occurred during the night and increased over the season from 8 mg g^{-1} DW in June to 27 mg g^{-1} DW in September, whereas the baseline levels of Glc decreased over the season from 77 mg g^{-1} DW in June to 53 mg g^{-1} DW in September. These changes balance each other out so that the baseline level of all three sugars combined was basically constant between July, August, and September, with June having a slightly higher baseline in total sugars than the other months (Fig. 4). Starch also had a clear diurnal pattern, peaking before the end of the light period (Fig. 3), with values between 42 and 47 mg g^{-1} DW, and reaching minimal values between 10.8 and 13.6 mg g^{-1} DW at predawn.

A more detailed analysis of August leaves by gas chromatography-mass spectrometry (GC-MS) showed that Suc was the only soluble carbohydrate (of the 17 most common soluble carbohydrates) to exhibit a diurnal trend (Supplemental Fig. S1). This finding justified using the results of the enzymatic assay for Suc, Glc, and Fru (in addition to starch) as a proxy for estimating diurnal changes in foliar carbon content in our export calculation.

Osmolality

To understand diurnal osmolality patterns, we measured the osmolality of fluid extracted from leaves frozen immediately after collection (native osmolality) and leaves allowed to hydrate fully prior to being frozen (hydrated osmolality). Native osmolality varied diurnally due to both passive dehydration and active solute accumulation (Fig. 5). The effect of dehydration is seen by comparing native and hydrated osmolality at midday; comparing predawn and midday hydrated osmolality shows the effect of solute accumulation. As expected, there was no difference in the native and hydrated osmolality at predawn (750 mmol kg^{-1}), and the seasonal peak in midday native osmolality was higher than the seasonal peak in midday hydrated osmolality (970 and 900 mmol kg^{-1}, respectively; Fig. 5). After converting from millimoles per kilogram to megapascals using the equation from Michel (1972; see "Materials and Methods"), the diurnal decrease in solute potential during August and September was ~−0.6

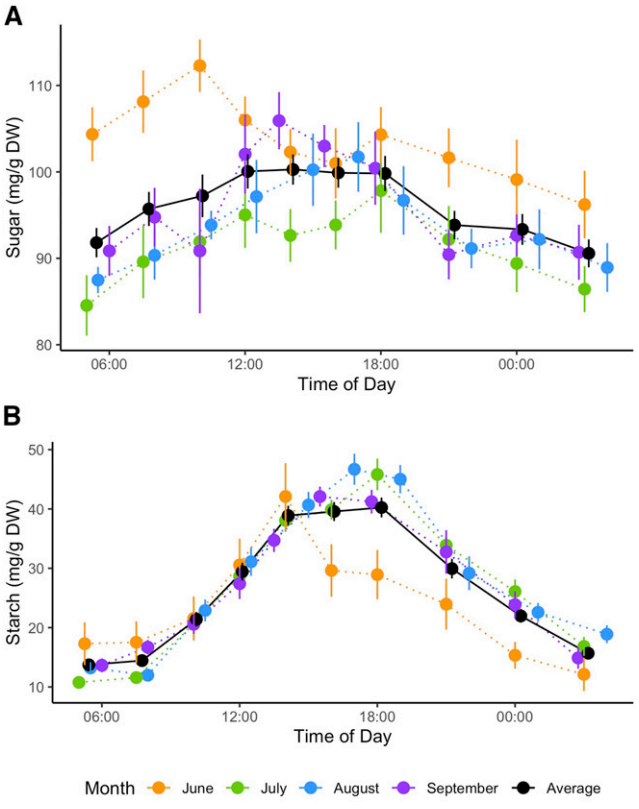

Figure 3. Diurnal patterns of total leaf sugars (Glc, Fru, and Suc) and starch for red oak leaves. Each monthly time point represents the average (± SE) of 15 leaves (three per tree) for sugars (A) and starch (B). The average over all months (± SE) is shown in black.

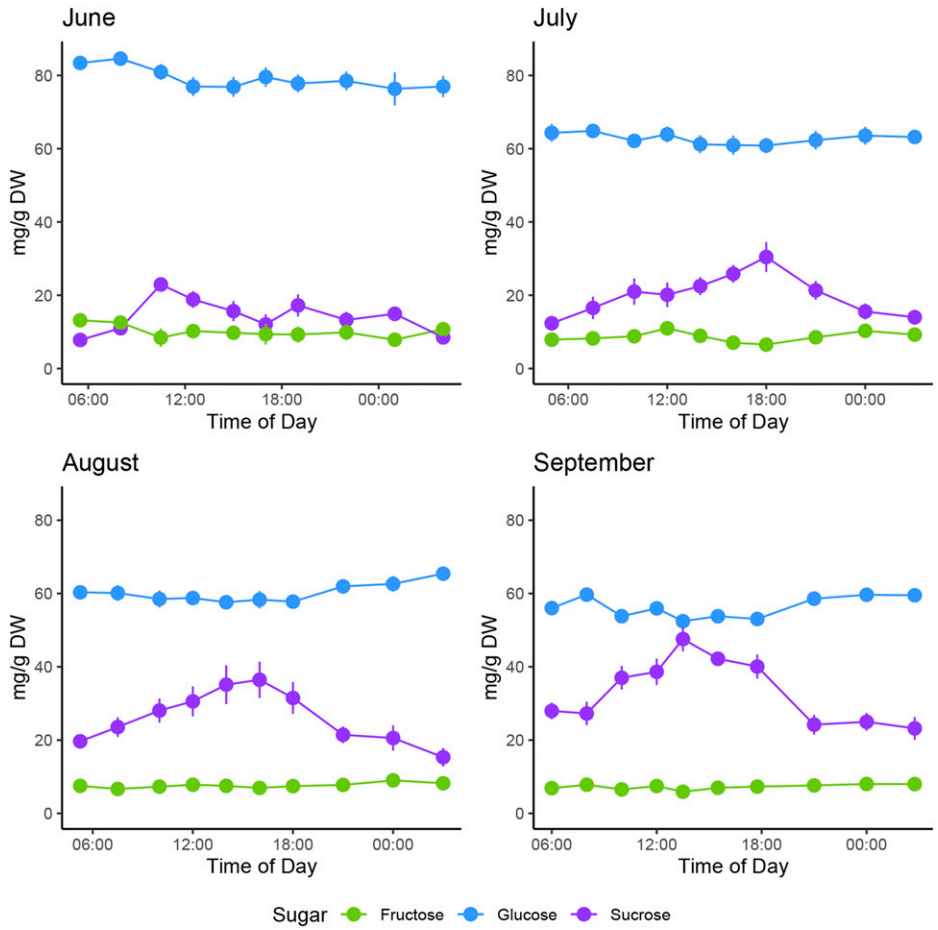

Figure 4. Diurnal and seasonal patterns of Suc, Glc, and Fru content of red oak leaves. Each point is the average (± SE) of 15 leaves (three per tree).

MPa (Fig. 6; Table 1). Of that diurnal change, ~25% was due to dehydration, ~40% was due to Suc, Glc, and Fru, and ~35% was due to other solutes (Table 1). The contribution of sugars to diurnal change in solute potential was calculated by converting milligrams per gram DW to millimoles per kilogram of water, and then to megapascals (see "Materials and Methods"). As for Ψ and A_N, there was a smaller diurnal change in osmolality in June compared with the other sampling months.

August and September are highlighted here because these months had the largest diurnal change in Ψ and thus are the months with the largest risk of turgor loss. If diurnal solute accumulation had not occurred in August and September, leaves would be within 0.3 to 0.5 MPa of turgor loss at midday. However, because these plants exhibit a diurnal change in solute potential, they maintain a turgor of at least 0.7 MPa (Fig. 7)

DISCUSSION

Export Patterns

Carbon export from the leaves of red oak trees tracks assimilation and appears not to be inhibited by diurnal fluctuations in water potential. Notably, export peaked when leaf turgor pressure was at its minimum. As this is the opposite of what would be expected, all else being equal and assuming passive loading, it requires some discussion. Whereas we cannot provide a definitive explanation, diurnal temperature variation could be important. In particular, respiration and growth may be lower during the night due to cooler temperatures, resulting in reduced sink demand and higher sink pressure. Because phloem movement is influenced by the difference in pressures between source and sink, this change could contribute to the patterns in export we observed. Temperature-related changes in phloem sap viscosity could also favor higher phloem flow rates during the day, even if there is lower pressure in the leaf.

With regard to passive loading, our finding that carbon export is inversely related to water potential raises the question of whether passive phloem loading can produce a Suc concentration in the phloem sufficient to drive bulk flow from source to sink. Answering this question, however, requires a way to measure phloem sap Suc concentration directly, as well as data about Suc compartmentalization in red oak leaves and red oak sink pressures. Absent these, we use cytoplasmic fraction data from the literature for a different oak

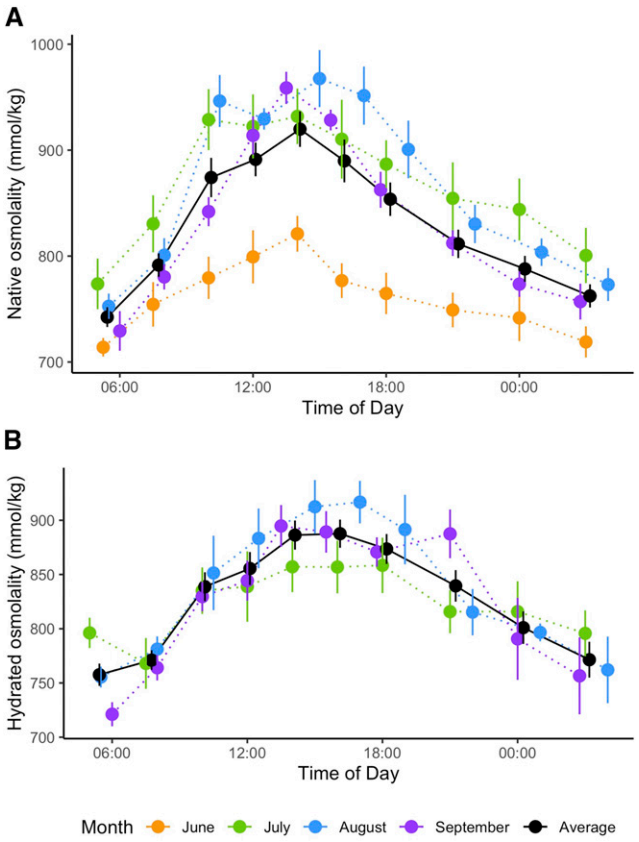

Figure 5. Diurnal patterns of native leaf osmolality and hydrated leaf osmolality for red oak leaves. Each monthly time point represents the average (± SE) of native leaf osmolality (A) and hydrated leaf osmolality (B) of five leaves (one per tree). The average over all months (± SE) is shown in black. June data were not collected for saturated osmolality due to methodological issues.

species, a model of passive loading parameterized for red oak, and sink (root) pressures for other species from the literature to understand whether, based on the Suc and water potential data from our study, it is possible to generate phloem pressure sufficient to move Suc from the leaves to the roots.

We begin by estimating the concentration of Suc in mesophyll cells, assuming that Suc is compartmentalized to the cytoplasm (i.e. excluded from the vacuole). In *Quercus robur* leaves, cytoplasm accounts for 21% (SD = 6.6%) of the total cell volume (Öner Sieben and Lohaus, 2014). Based on our data (average peak of 42 mg g^{-1} of Suc between 12 PM and 4 PM in September converted to 110 mmol kg^{-1} of water using measured relationships between leaf area, DW, and fresh weight) and a cytoplasmic fraction of 21%, we estimate the mesophyll cytoplasm Suc concentration to be 520 mmol kg^{-1} (1 SD range of 400–760). The SD values presented here, and below, reflect the uncertainty regarding the cytoplasmic fraction reported for *Q. robur* leaves (Öner Sieben and Lohaus, 2014).

Next, we estimated Suc concentrations in the phloem based on a transport model parameterized for passive loading in red oak leaves (Rockwell et al., 2018). Because red oak is hypostomatous, a difference of only ~10 mmol kg^{-1} is necessary to drive diffusion from the furthest palisade mesophyll cell to the phloem (Rockwell et al., 2018), giving an estimated Suc concentration in the phloem of 510 mmol kg SD (1 SD range of 390–750; Fig. 8). In the spongy mesophyll, a larger Suc gradient (~200 mmol kg^{-1}) would be needed to overcome convection due to water movement to the stomata. Although we do not know how bulk leaf Suc is divided between the two tissues, in red oak the palisade dominates the mesophyll volume, and therefore we will continue our inferences based on the palisade estimates. Notably, these phloem Suc concentrations are in the realm of the few reported phloem Suc concentrations for red oak (e.g. ~500 mmol kg^{-1}; Münch, 1930).

Lastly, we calculate what pressure difference between the source and the sink could be generated by this phloem Suc concentration. Assuming Suc represents ~85% of the total osmolality in the phloem (Pate, 1976), we estimate total phloem sap osmolality of 600

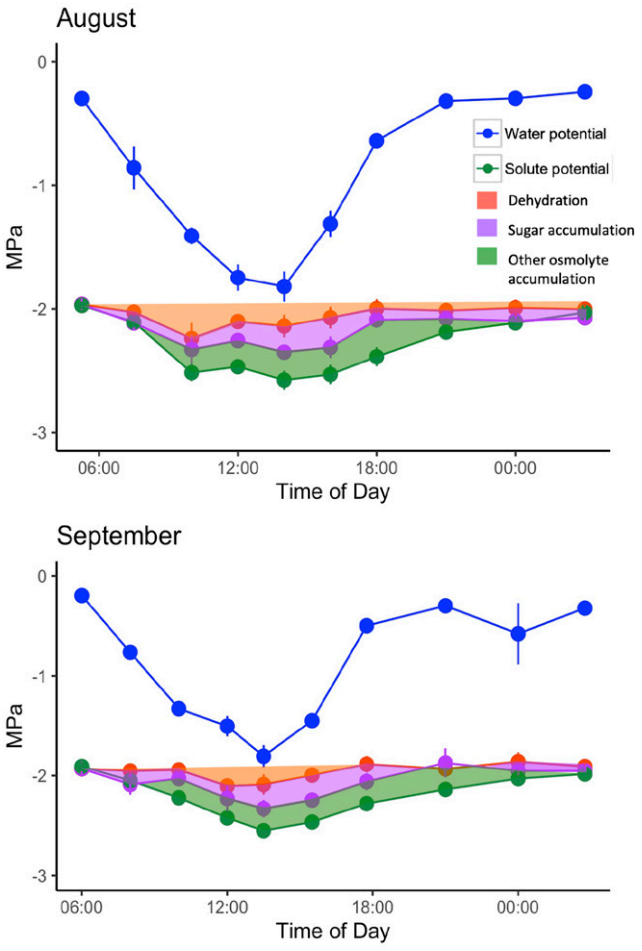

Figure 6. Diurnal patterns of water potential and solute potential for red oak leaves. The different components contributing to the diurnal change in solute potential are dehydration (orange), sugar accumulation (purple), and other osmolyte accumulation (green).

Table 1. *Factors contributing to the average midday decrease in leaf osmotic potential between 12 PM and 5 PM*

Month	Average Osmotic Shift	Dehydration	Sugar Accumulation	Accumulation of Other Osmolytes
	MPa		*MPa (%)*	
August	−0.62	−0.17 (28%)	−0.26 (42%)	−0.18 (29%)
September	−0.57	−0.13 (23%)	−0.21 (37%)	−0.23 (40%)

mmol kg^{-1} (1 SD range of 460–880). Here, the ±1 SD range overlaps with what has been reported for red oak phloem osmolality (875 mmol kg^{-1}; Hammel, 1968). Notably, the reported osmolality (Hammel, 1968) and Suc concentrations (Münch, 1930) are not compatible with each other, suggesting that either there is a large variation in either of these parameters and/or there is a large variation in the ratio of Suc concentration to total osmolality. Our calculated phloem sap osmolality corresponds to a solute potential of −1.6 MPa (1 SD range of −1.2 to −2.3; Fig. 8). In September, the minimum xylem water potential in the minor veins is −1.7 MPa, based on models of water flow through red oak leaves that indicate minor vein xylem to be 0.1 MPa more hydrated than bulk leaf water potential (Rockwell et al., 2014). Although our average solute potential would not generate positive pressure in the phloem, the upper bound of the 1 SD range corresponds to a pressure of 0.6 MPa (Fig. 8). Assuming sink phloem pressure is between 0.4 and 0.7 MPa, based on data from species other than red oak (Rygol et al., 1993; Pritchard, 1996; Knoblauch et al., 2016; Savage et al., 2017), a maximum pressure difference between the source and the sink would be 0.2 MPa. If the location of the sink is taken as the base of the tree, the addition of an ~0.2 MPa change in gravitational potential (20 m) would create ~0.4 MPa of driving force from source to sink.

To return to the question of whether our findings support the idea that passive phloem loading can produce a concentration in the phloem sufficient to drive phloem transport from source to sink, the answer is "possibly". The phloem solute concentrations we estimate from our measurements of bulk leaf Suc concentration and data from the literature are narrowly sufficient to draw water in from the xylem and generate enough pressure to drive flow to the sinks, even with the conservative assumption of complete compartmentalization of the Suc to the cytoplasm. The few studies that have measured Suc compartmentalization suggest that it is not exclusively confined to the cytoplasm (Öner-Sieben and Lohaus, 2014; Fink et al., 2018). Therefore, our findings could be interpreted as providing support for a model characterized by mixed (Slewinski et al., 2013) or active loading for red oak, instead of purely passive loading, which would help drive up the Suc concentration in the phloem. However, our estimates make many assumptions (e.g. that distribution of Suc between the palisade and spongy mesophyll cells is equal or that Suc is completely compartmentalized to the cytoplasm), because their main purpose is to sketch out whether our findings fit within the Münch hypothesis and passive loading framework. The lack of data regarding cytoplasmic Suc concentrations and phloem Suc concentration impedes us from obtaining a robust picture of this loading and transport process. New advancements in visualizing Suc in plants (Guendel et al., 2018) raise the opportunity to further validate and quantify Suc compartmentalization in different cellular compartments in order to better understand the mechanism of the loading and transport process.

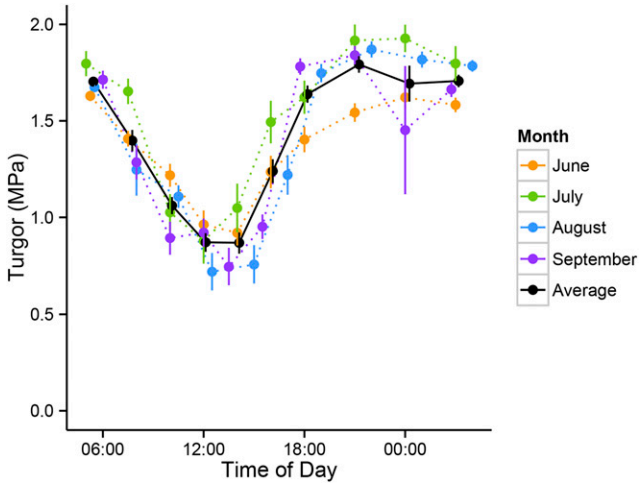

Figure 7. Diurnal patterns of leaf turgor for red oak leaves. Leaf turgor was calculated as the difference between water potential and solute potential (± SE).

Finally, assuming that energy is needed either to achieve compartmentalization or drive Suc transporters, why not export at night when relaxed Ψ would lower the energy required to load a sufficient concentration of sugars in the phloem? There are four potential reasons (1) starch production is limited by available space in the cell due to its confinement to plastids; (2) the rate of starch production is already at its maximal values; (3) the cost of starch production is higher than the cost of concentrating or exporting the sugars; or (4) the demand of other tissues requires export to occur during the day. The first two possibilities are the least likely, because in experiments where sinks were removed, starch content increased in the leaves, suggesting that it is not at a maximum level with regard to space or production rate during normal functioning (Schulze et al., 1991). Therefore, we hypothesize that some combination of the cost of starch production and the demand for carbon in other parts of the plant may be the cause of sugar export during the day. In addition, the phloem transports a range of metabolites, as well as

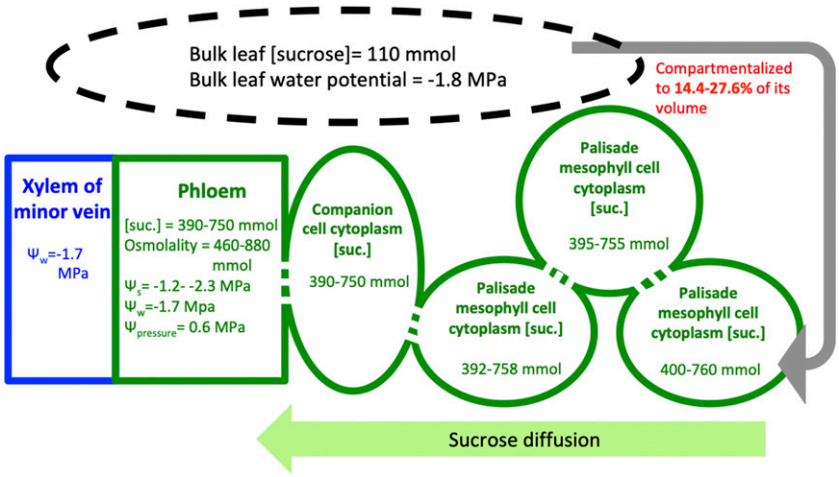

Figure 8. Diagram of the estimated prephloem Suc diffusion pathway for red oak leaves. Both the concentrations and the phloem pressure that could be generated due to complete compartmentalization of Suc to the cytoplasm in the mesophyll are shown. Our measurements of bulk leaf Suc and water potential, in addition to data and models from the literature, were used to estimate cytoplasmic Suc concentrations, the concentration gradient necessary to drive diffusion through the mesophyll to the phloem, and the subsequent Suc concentration, total osmolality, and pressure of the phloem (shown here as the maximum of the range).

hormones and other signaling molecules (Hoad, 1995), and it may be necessary for sugar export to occur during the day to maintain transport of these compounds.

Carbohydrate Levels and Osmotic Adjustment

For sugars, and specifically Suc, we observed a diurnal pattern in leaves of mature red oak trees, which is consistent with the findings of Dickson (1987), Klages et al. (2001), and Faria et al. (1996) for trees. Suc increased in concentration over the season both in baseline concentration (as observed in Passarinho et al., 2006) and diurnal amplitude. Other studies on the metabolites of oak trees (*Quercus prinus* [Gebre and Tschaplinski, 2002] and *Quercus suber* [Passarinho et al., 2006]) found a range of sugar compositions in oak leaves similar to our findings. With respect to starch, the commonly observed trend of starch peaking before the end of the light period was observed. The proposed reason for this is that the starch is broken down and exported over the course of the night (Fondy and Geiger, 1982; Tixier et al., 2018).

It is important to note that between our two sugar assays (GC-MS and enzymatic), different quantities of Glc were observed (20 versus 60 mg g^{-1}; Fig. 4; Supplemental Fig. S1). A potential reason for this discrepancy is the difference in extraction methods, which can influence the sugar value (Quentin et al., 2015; Landhäusser et al., 2018). Our analysis of Suc, Glc, Fru, and starch was completed with the enzymatic method that employs the recently suggested extraction approach outlined in Landhäusser et al. (2018). Additionally, because neither method found a diurnal pattern in Glc, our export calculations and solute potential diurnal dynamics calculations would be similar using the two measurements.

Temporal changes in solute potential have been observed on both a seasonal and a diurnal time scale (Hinckley et al., 1978; Davies and Lakso, 1979; Roberts et al., 1980; Bowman and Roberts, 1985; Marigo and Peltier, 1996; Patakas and Nortsakis, 1999; Dichio et al., 2003; Paljakka et al., 2017), although sometimes a diurnal change is not observed (e.g. Lazzarin et al., 2019). Our data show that for red oak there is a diurnal change in solute potential and that Suc is a large component, with increasing importance throughout the season. On average, over one-third of the observed daily shift in solute potential can be attributed to Suc with approximately another third being due to dehydration. The last third of the daily shift in solute potential is likely due to the accumulation of inorganic ions. GC-MS results showed that the other major organic compounds in the leaf do not have strong diurnal patterns, suggesting that inorganic ions play a key role in osmotic adjustment. In support of this, ions were observed to be a large component of osmotic adjustment in many species (Marigo and Peltier, 1996; Warren et al., 2012; Degu et al., 2019).

The finding that sugar plays a large role in diurnal changes in solute potential highlights an important role of sugars in leaf functioning. These turgor-loss buffers may be important in maintaining assimilation and avoiding stress responses (Bowman and Roberts, 1985). Furthermore, in times of hot, dry winds or sunflecks, Ψ could decrease by 0.3 to 0.6 MPa (Young and Smith, 1979). For the leaves in this study, if such a perturbation was experienced in August and September and no Suc was accumulated in the leaf, the leaf would be at risk of losing turgor. Interestingly, however, if this Suc was instead a hexose, it would have double the impact on osmolality and solute potential (i.e. it would increase the leaf's turgor buffer even more, because for the same amount of carbon, hexoses have double the molar concentration). This suggests that whereas sugars are an important component of diurnal changes in solute potential, the buildup of Suc is more related to diffusion and phloem function than to osmolality.

Diurnal fluctuations in sugars, starch, and osmolality imply that time of day is important when sampling for these traits and taking measurements that depend on these traits, such as pressure-volume curves. Based on this study, if sampling occurred at predawn for a pressure-volume curve (and was rehydrated), the

turgor loss point could be underestimated by up to 0.44 MPa (the active component of the observed diurnal shift in solute potential). This is large in comparison to the safety margin for turgor loss point estimated for many trees in nature. For example, *Betula papyrifera*, *Acer negundo*, *Ulmus parvifolia*, *Robinia pseudoacacia*, and *Malus baccata* were reported to have differences between Ψ at midday and Ψ at turgor loss point of 0.52, 0.54, 0.72, 0.65, and 1.15 MPa, respectively (Ranney et al., 1990), based on samples collected at predawn. If these trees exhibit the same amount of osmotic adjustment during the day as red oaks, the safety margin for turgor loss at midday would be almost double that for red oak in some scenarios. This suggests that some of the previously measured small or negative safety margins between Ψ at midday and Ψ at turgor loss point may need to be revisited.

CONCLUSION

Our findings highlight the fact that, despite negative Ψ at midday, the phloem is capable of exporting carbon from red oak leaves. Additionally, diurnal accumulation of NSCs assists in turgor maintenance, elucidating a key role of NSCs in leaf functioning. These findings shed light on how phloem and carbon dynamics function in times of varying water availability and show that over the course of the day, the local accumulation of sugars accompanying photosynthesis plays an important role in maintenance of turgor pressure in both the leaf mesophyll and the phloem.

MATERIALS AND METHODS

Site and Tree Description

Five mature (>20 m) red oak (*Quercus rubra*) trees at Harvard Forest in Petersham, Massachusetts, were selected for study due to their canopy dominance and south-facing orientation. Sampling occurred over 24 h at four different seasonal time points in the 2017 growing season: June 11 to 12, July 4 to 5, July 30 to 31, and September 11 to 12 (the July 30–31 sampling is referred to as "August" in the figures and text). The dates were selected due to minimal cloud cover. The average and maximum photosynthetically active radiation (PAR; μmol m^{-2} s^{-1}) during the day were 679 and 1,688 (June 11), 722 and 1,779 (July 4), 954 and 1,746 (July 30), and 944 and 1,772 (September 11), respectively. During each 24-h period, sampling occurred 10 times (every 2–3 h).

Field Measurements and Sample Collection

For each tree, at each time point, net assimilation (A_N) was measured on four sunlit leaves using a commercial gas exchange system (LI-6400, LiCor). In the cuvette, light, relative humidity, and temperature were all held at ambient environmental conditions. The red-blue light source inside the chamber was manually set to the PAR the specific leaf was receiving, which was measured using the LI-6400 PAR sensor. The leaves were then removed from the tree; three of them were immediately sealed in plastic bags and placed on dry ice, and the fourth was placed in a humidified plastic bag in the dark to be brought back to the lab for Ψ measurements (within 2 h of collection).

Fresh weight and then Ψ was measured using a pressure chamber (Soil Moisture Equipment) on the nonfrozen leaf. Next, its petiole was placed in water for 10 min to become saturated. We found that after 10 min the water potential of the leaves was 0 to 0.2 MPa below the atmospheric pressure (not reported).

Leaf area was measured for all leaves using a leaf area meter (LI-3000, LiCor). Leaves were stored in a −80°C freezer until they were further processed for NSCs and osmolality.

NSCs and Export

The concentrations of starch and three sugars (Suc, Glc, and Fru) for three leaves per tree per sampling time point were quantified using a modified protocol of the Megazyme starch and Sigma Fru assay kits used by Gauthier et al. (2014) and further described in the Supplemental Materials and Methods. Briefly, leaves were freeze dried, weighed, and ground (excluding the midrib). Ethanol was then used to extract the sugars from 5 mg of the dry and ground sample. Using a plate reader (SpectraMax i3) reading at 340 nm, the Sigma Aldrich Glc assay reagent phosphoglucose isomerase and invertase were used to break down and measure Glc, Fru, and Suc, respectively, as Glc equivalents in duplicate samples. Sugar values were calculated based on Glc, Fru, and Suc standard curves. Peach standards were used for additional validation (NIST SRM 1547, Sigma-Aldrich). For starch, α-amylase and amyloglucosidase were used, along with the Megazyme GOPOD reagent, to identify starch, again in duplicate samples, using a cuvette reader (100 UV-Vis Spectrometer, Cary) to measure absorptance at 515 nm. Starch values were calculated based on a pure Glc standard curve, and a standard of pure starch was also used.

Additionally, for one leaf per tree, five times over the course of the day during the August sampling time point, GC-MS analysis was used to identify the most common organic compounds in the leaf (catechine, inositol isomer 2, gallic acid, malic acid, maltose, ribose, shikimic acid, deoxyribose, Gal, maltose, myoinositol, Suc, Glc, Fru, quinic acid, inositol isomer 1, and chlorogenic acid) and their diurnal patterns. Leaves were freeze-dried and ground. The analysis procedure followed the Small Molecule Mass Spectrometry protocol of the science core at Harvard University. For extraction, 2 mL of methanol and 4 mL of chloroform were added to 2 mg of each sample, followed by 2 mL of water with 4 μ_M ^{13}C6 Glc as an internal standard. The samples were then sonicated for 1 min and centrifuged at 4°C at 2,000 rpm for 10 min (Centrifuge 5414 C and 18-place rotor F-45-18-11; Eppendorf). Then, 1.5 mL of the supernatant was dried under N_2 in an autosampler vial and 100 μL of methoxyamine (10 mg mL^{-1} in pyridine) was added to each sample. Following incubation at 40°C for 3 h, 100 μL of N,O-Bistrifluoroacetamide +1% (w/w) trimethyl chlorosilane was then added to each sample, and they were reincubated at 37°C for 30 min. The samples were then transferred to micro inserts and injected into the GC-MS (GC-Q-Exactive, Thermo Fisher). Peaks were manually analyzed using Quan Browser.

Export was calculated using a mass balance approach (Quick et al., 1992; Rogers et al., 2004; Davey et al., 2006):

$$\text{Export} = \frac{A_n + A_{n-1}}{2} - \frac{C_n - C_{n-1}}{T_n - T_{n-1}} \quad (1)$$

where Export is expressed as μmol m^{-2} s^{-1}, A is the net assimilation at a specific time point n (μmol m^{-2} s^{-1}), C is the carbon in the NSC measurements calculated by multiplying the mg g^{-1} sugar quantity by the weight fraction of carbon in each molecule and then dividing by the molar weight of carbon and converting the quantity to an area basis using measured DW and leaf area relationships (μmol m^{-2}), and T is the time of measurement (s). This method has been shown to agree with alternate methods based on DW export quantification (Terry and Mortimer, 1972) and isotopes (Leonardos et al., 2006). However, the mass balance approach becomes increasingly difficult at transitional times of day (e.g. sunrise and sunset), because net assimilation rates are changing quickly and averaging between two time points becomes increasingly less accurate.

Whereas there has been debate about the accuracy of NSC methods for absolute quantities (Quentin et al., 2015), a recent study has shown that an enzymatic assay with freeze drying and ethanol extraction, as performed here, is capable of precisely and accurately quantifying NSCs in leaves (Landhäusser et al., 2018). That said, in one of the three experiments in Landhäusser et al. (2018), the enzymatic assay showed a 5% underprediction for a synthetic sample composed of compounds that are commonly found in plant tissues. We note, however, that a 5% underprediction for our sugar and starch measurements would not change the reported carbon export patterns in regard to their tracking with assimilation.

Leaf Solute Potential

Osmolality was measured on half of the frozen saturated leaf (July, August, and September) and on half of a leaf that was frozen immediately after being

removed from the tree (all months) to quantify both the solute potential due to osmotic accumulation in a hydrated leaf and the native solute potential of the leaf. The leaves were thawed and hand ground in an Eppendorf tube with a 0.22-μm pore cellulose acetate membrane-containing filter (Costar 8161, Corning). The tubes were then centrifuged for 10 min at 12,000 RPM (occasionally spun for an extra 5 min to extract more liquid; Microfuge 18 and F241.5P Rotor, Beckman Coulter). Ten microliters of the liquid collected at the bottom of the centrifuge tube after centrifuging was then pipetted onto a filter paper disc and the osmolality was determined using a vapor pressure osmometer (Wescor).

The following equation from Michel (1972) was used to calculate solute potentials from millimole per kilogram concentrations:

$$\psi_s = -(0.089m^2 + 0.998m) \times (DRT \times 1,000) \quad (2)$$

where ψ_s is the solute potential (MPa), m is the osmolality measurement (mol kg^{-1}), D is the density of water as a function of temperature T (0.998 g m^{-3}), R is the gas constant (8.3143×10^{-6} MPa m^{-3} mol^{-1} K^{-1}), and T is temperature (298 K). Apoplastic water content was included in our measurements of osmolality because both apoplastic space and symplastic space were ground together. However, only the osmolality of the symplast is relevant in terms of the turgor pressure of NSC accumulation. To account for this, we adjusted the concentration based on the assumption that the apoplast does not contribute any solutes and that it makes up 10% of the leaf's water-filled volume (average value in Tyree, 1976).

Dehydration, sugar accumulation, and other osmolyte accumulation as a component of diurnal changes in solute potential were calculated as follows. Dehydration was calculated as the difference in osmolality between the hydrated osmolality and the native osmolality. This difference was then converted from millimoles per kilogram to megapascals using Equation 2. The difference was then added to the predawn solute potential of the native solute potential to observe the contribution of diurnal change in native solute potential due to dehydration. Sugar accumulation was calculated as the change between predawn sugar concentration and all of the other sampling time points. This change in concentration was then converted to millimoles per kilogram using measured relationships between DW, fresh weight, and leaf area. This millimoles-per-kilogram amount was then converted to a solute potential through use of Equation 2. This change in megapascals was added to the change in solute potential due to dehydration in order to visually observe the contribution of diurnal change in native solute potential due to sugar accumulation. Lastly, the contribution of other osmolyte accumulation to the diurnal change in native solute potential was the part of the change in native solute potential not due to either dehydration or sugar accumulation.

Statistical Analyses

All statistical analyses were performed in R 3.1.1 (R Core Team, 2014). To understand the effect of time of day, month, and type of tree on studied plant traits, one-way ANOVAs were performed with time of day, month, and tree as fixed factors. Significant factors for all ANOVAs are shown in Supplemental Table S1.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Diurnal patterns of the 17 most common organic compounds in red oak leaves.

Supplemental Table S1. Significant factors in one-way ANOVAs.

Supplemental Materials and Methods.

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