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Osmotic regulation mediates embolism vessel refilling via bark water uptake in *Salix Matsudana*

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Abstract: The aim of this study was to understand the role of bark water uptake in xylem embolism vessel refilling in *Salix matsudana*. Isolated branch segments of *Salix matsudana* were soaked in deionized water. Next, the percent loss of conductivity (PLC), the volume, the osmotic potential (Ψs), the concentrations of soluble sugar and ions in xylem sap and the concentration of NSC in the xylem were measured after 2, 4 and 6 h. The PLC decreased, and the volume of xylem sap increased, compared with initial values after soaking for 2, 4, and 6 h. Moreover, the osmotic potential (Ψs) of xylem sap decreased. The concentrations of ions and soluble sugar in the xylem sap increased significantly compared with the initial concentrations after soaking. Based on our findings, water not only entered the xylem vessels through the bark but also repaired the embolism of branches in a certain time. These findings will contribute to the understanding of the physiological and molecular mechanisms between bark water uptake and embolism repair.

Keywords: Bark water uptake; Embolism repair; Osmotic regulation

1. Introduction

Drought stress leads to an increase in xylem tension. The gas in the surrounding interstitial invades xylem conduits through the pit membrane. The bubble in the conduits expands rapidly under negative pressure, fills the entire tube cavity, and induces embolism[1]. Embolism destroys the water delivery function of the vessel, reduces the hydraulic conductivity of xylem, leads to stomatal closure of leaves, blocks photosynthesis and weakens productivity[2]. In severe cases, embolism causes the death of the entire plant[3]. People have long speculated that xylem embolism repair is driven by root pressure. However, recent studies find that under drought stress, root pressure is only 0.1 MPa, and the height of the water column in the driving conduit is less than 10 m, which has very little recovery effect on xylem embolism in distal tissues of tall trees. The formation of root pressure requires a low transpiration rate and high soil available water, and pressure can also be easily limited by root activity[4]. This regulatory mechanism is common in herbaceous plants, but only a few woody angiosperms use root pressure to repair embolism[5]. Numerous experiments demonstrate that embolization occurs frequently in the process of plant water transport and is not restricted by space-time factors. Therefore, synchronous repair in situ is very important to maintain the continuity of water conduction and then to ensure gas exchange of the leaf. In recent years, more experiments provide evidence of xylem embolism repair under negative pressure, advancing a "novel refilling" embolism repair mechanism[6]. According to these experiments, the consumption of carbohydrates stored in xylem parenchyma cells causes the radial movement of sugar and water in the phloem through the xylem ray cells, forming different osmotic pressure around the embolism vessels. The establishment of osmotic differential pressure is the primary driving force to drive water flow to embolism vessels to complete water refilling.

However, the water transport efficiency, the ability to repair embolism and the hydraulic isolation mechanism may be different between coniferous and broadleaf species because of their different xylem water transport structures[7]. Particularly after the leaves of deciduous trees fall off in winter, whether the radial transport of water participates in embolism repair through direct water absorption is unknown. If deciduous species can repair embolism by directly absorbing water from bark, identifying the key genes that are involved in and responsible for the radial water supply in the embolism repair process is essential, in addition to the regulation approaches of radial water transport. We speculated that bark plays a key role in the repair of xylem embolism throughout the growth period, particularly for deciduous species in the leafless stage. Based on the theoretical system of embolism repair driven by osmotic regulation, bark

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may regulate and control the starch response of woody parenchymal cells and transmembrane transport of osmotic adjustment substances by providing non-structural carbohydrates for woody parenchymal cells and then increase the transposition of osmotic adjustment substances to embolism conduits, reduce the osmotic potential of embolism conduits and drive water to inject into embolism conduits from surrounding parenchymal cells.

Salix matsudana is not only an important fast-growing timber but is also a type of tree for farmland protection and soil and water conservation in northern China. The tree has strong adaptability to drought, water flooding, salinization and other difficult site conditions. Moreover, *Salix matsudana* has high greening, ornamental and economic value. In this study, embolized branches with 50% conductance loss were used as the experimental material. Furthermore, the percent loss of conductivity (PLC), volume, osmotic potential (Ψs), the concentrations of soluble sugar and ions in xylem sap and the concentration of NSC in the xylem were measured after 2, 4 and 6 h.

2. Materials and methods

2.1 Plant materials and treatments

Plant materials used in this study were collected from Salix matsudana (~4 m height and ~0.1 m diameter), which openly grow at the experimental base of the Chinese Academy of Forestry. Small branches (~1 m length and 5-10 mm diameter) were randomly selected from low hanging limbs (~2.5 m above the ground). The selected branches had over 30 cm long segments with no leaves, which ensured the full coverage with bark. Moreover, branches were collected from limbs facing the same aspect around the trees. The branches were naturally dried to an embolism degree of P50 on an experimental table (according to preliminary experimental results, the P50 of Salix matsudana was -1.9 ± 0.05 MPa).

We excised six branches and cut each branch into four segments (50 mm length) under water (the number of segments from the upper to the lower end of the morphology was I–IV). Segment I was the initial segment, whereas the segments II, III and IV were the treated segments. Both ends of segments II, III and IV were quickly sealed with a covering of siliconized acrylic caulk. Then, the segments were placed in a sink containing deionized water (Mason et al. 2016). Specifically, segment II was removed after 2 h, segment III was removed after 4 h and segment IV was removed after 6 h. During the treatment, the saturated water was exchanged every 2 h. Six independent biological replicates were analysed for each treatment.

2.2 Analysis of branch segment biomass

From the collected branches, we cut segments (50 mm length) under water, quickly sealed both ends with a covering of siliconized acrylic caulk and placed the segments in a sink containing deionized water for 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h. Then, we removed each submerged branch segment, patted it with a paper towel to remove surface water, recorded the weight and returned the branch segment to the water bath.

2.3 PLC

The measurement of PLC was as follows. Branch segments were selected with the length of 50 mm. The two ends of a segment were cut flat with a single blade under water, and the water conductivity of the segment was measured with a 25 $mmol \cdot L^{-1}$ KCl solution filtered through a 0.4 μm microporous membrane. The initial water conductivity was measured using the pressure of 4 kPa to push the solution through the stem segment. Then, the segments were flushed with 0.1 MPa pressure for 30 s to wash out all bubbles in the segment (according to preliminary experimental results, all embolisms in a segment were removed at 0.1 MPa pressure for 30 s). Furthermore, the maximum water conductivity was measured using the pressure of 4 kPa. The percent loss of conductivity was calculated as PLC (%) = (1-Ki/Kmax) × 100%, where Ki refers to the initial conductivity and Kmax refers to the maximal conductivity.

2.4 Volume of xylem sap

The xylem sap was extracted by the centrifugal method. We removed the bark, the segment was immediately spun at 12,000 rpm in a centrifuge to remove the xylem sap, and then ~3 mm was cut off the

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segment, which was spun again; this procedure was repeated until the entire segment was cut and spun.

2.5 Osmotic potential of xylem sap

Osmotic pressure was determined with an Osmomat 030 (Gonotech, Germany). The osmotic potential was calculated as Ψs = -i CRT, where i is the dissociation coefficient of the solute, C is the osmotic pressure, R is the gas constant, and T is the thermodynamic temperature.

2.6 Concentration of ion and soluble sugar in xylem sap

Because K^+, Ca^{2+}, Na^+ and Mg^{2+} were the primary inorganic ions in xylem vessels, we used the sum of the concentrations of K^+, Ca^{2+}, Na^+ and Mg^{2+} as the total concentration of xylem sap ions. Xylem sap, 50 μL, was extracted by the centrifugal method, and the volume was fixed to 5 mL with deionized water. The concentrations of K^+, Ca^{2+}, Na^+ and Mg^{2+} were determined using an inductive coupling plasma emission spectrograph (iCAP6300ICP-OES Spectrometer; Thermo Scientific, Waltham, MA, USA). The concentration of soluble sugar was measured with a Plant Soluble Assay Kit (Solarbio, China).

2.7 Concentration of starch and soluble sugar in xylem cell

Xylem, 0.1 g, was extracted. The concentration of soluble sugar was measured with a Plant Soluble Assay Kit (Solarbio, China). The concentration of starch was measured with a Plant Starch Assay Kit (Solarbio, China).

2.8 Statistical analysis

For statistical analysis, data were processed using analysis of variance (one-way ANOVA). The differences among treatments were assessed using Tukey's test with the SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL, USA). Differences between treatments were considered to be significant at $P < 0.05$.

3. Results

3.1 Water enters xylem through bark

In all tested branches, segment mass increased with soak time over a period of 24 h. Percent increase in branch mass changed most rapidly over the first 4 h, and stem mass increased 100.79 mg. Over a period of 24 h, stem mass increased 134.37 mg compared with the initial weight (Fig. 1a). This result is consistent with that of research on coastal redwood crowns and showed that bark could absorb water. The initial ratio of xylem sap volume of the segments was 0.13 ± 0.001 $\mu L \cdot mm^{-3}$, which increased by 21.51, 20.29 and 23.75% compared with the initial ratio after soaking for 2, 4 and 6 h, respectively (Fig. 1b). Thus, we argue that bark absorbed external water, and a fraction of water was transported into the xylem vessels. This transport provided a source of water for the repair of the embolized vessels. The initial PLC of the segments was 50 ± 2%, which decreased by 30.85, 21.24 and 34.37% compared with the initial PLC after soaking for 2, 4 and 6 h, respectively (Fig. 1c). This result is consistent with the phenomenon of xylem refilling studied using visualization techniques such as MRI, microCT scanning, experimental manipulations and modelling. The initial Ψs of xylem sap was -0.07±0.05 MPa, which decreased by 10.00, 22.79 and 87.18% compared with the initial Ψs after soaking for 2, 4 and 6 h, respectively (Fig. 1d). Combined with the results for PLC and xylem sap volume, we considered that the osmotic potential difference between conduits might be the driving force to promote bark water uptake.

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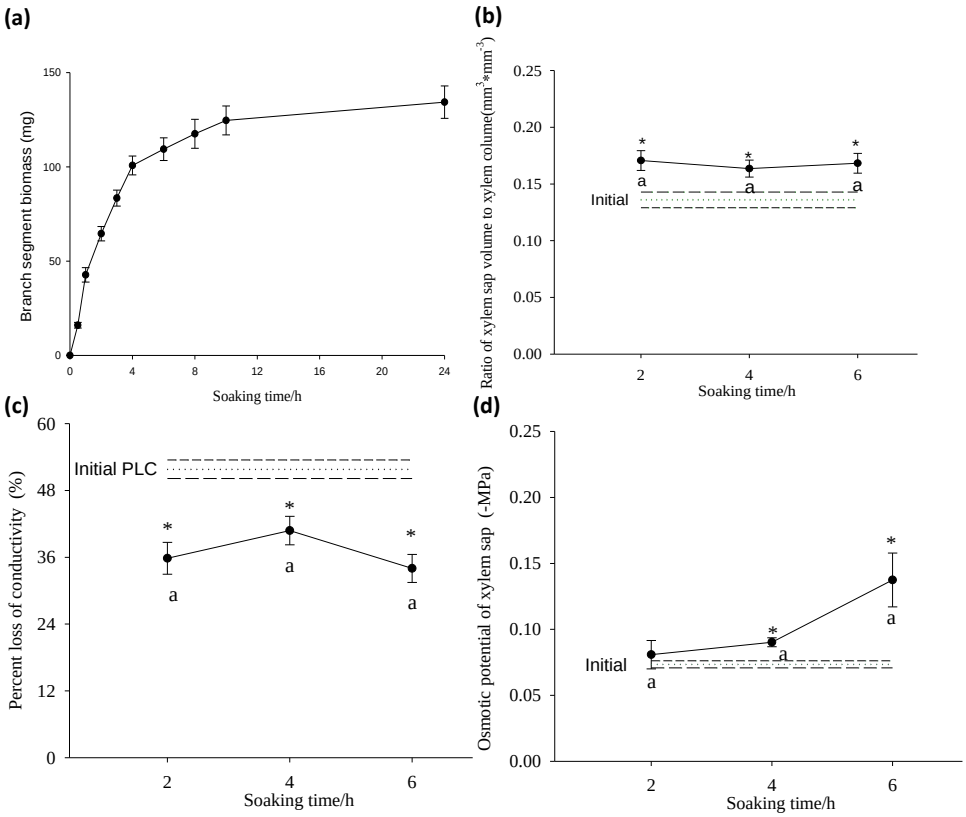

Fig. 1 Mass increment of branch segment (a), ratio of xylem sap volume to xylem volume (b), PLC (c) and osmotic potential of xylem sap (d) at different soaking times. Data in the figure are the mean ±SE (n=6). The dotted line represents the average initial value of the segment (before soaking in water); the dashed line indicates the initial mean ±SE (n=6). Different lowercase letters denote significant differences (P < 0.05) between soaking times; ∗ indicates significant differences (P < 0.05) between initial and after soaking values.

3.2 Change of osmotic regulating substance

The biology of the refilling process has focused on studies of xylem parenchyma cells that are assumed to provide both water and energy in response to refilling treatments[8]. Our study found that the concentration of soluble sugar in xylem sap increased by 12.86, 24.67 and 39.98% compared with the initial concentration after soaking for 2, 4 and 6 h, respectively (Fig. 2a). The concentration of soluble sugar in the xylem increased by 25.52, 42.07 and 33.33%, and the concentration of starch in the xylem decreased by 3.77, 4.96 and 10.24% compared with the initial concentrations after soaking for 2, 4 and 6 h, respectively (Fig. 2b, c). The consumption of carbohydrates in xylem parenchyma cells is considered by some to create a strong signal for phloem unloading and leads to the sugar and water in the phloem to move radially through the xylem rays. However, little research has been conducted on the role of ions in the refilling of embolized vessels. Because K^+, Ca^{2+}, Na^+ and Mg^{2+} were the primary inorganic ions in xylem vessels, we used the sum of the concentrations of K^+, Ca^{2+}, Na^+ and Mg^{2+} as the total concentration of xylem sap ions. In our study, the concentration of ions increased by 30.25, 48.80 and 46.00% compared with the initial concentration after soaking for 2, 4 and 6 h, respectively (Fig. 2d). In fact, we demonstrated that osmotic regulation repaired embolism by mediating bark water uptake. However, the fate of the osmotic regulating substance is unknown. Additionally, we cannot conclude whether the substance is used for respiration or transportation in cells. Overall, the role of this substance in the refilling process requires elucidation.

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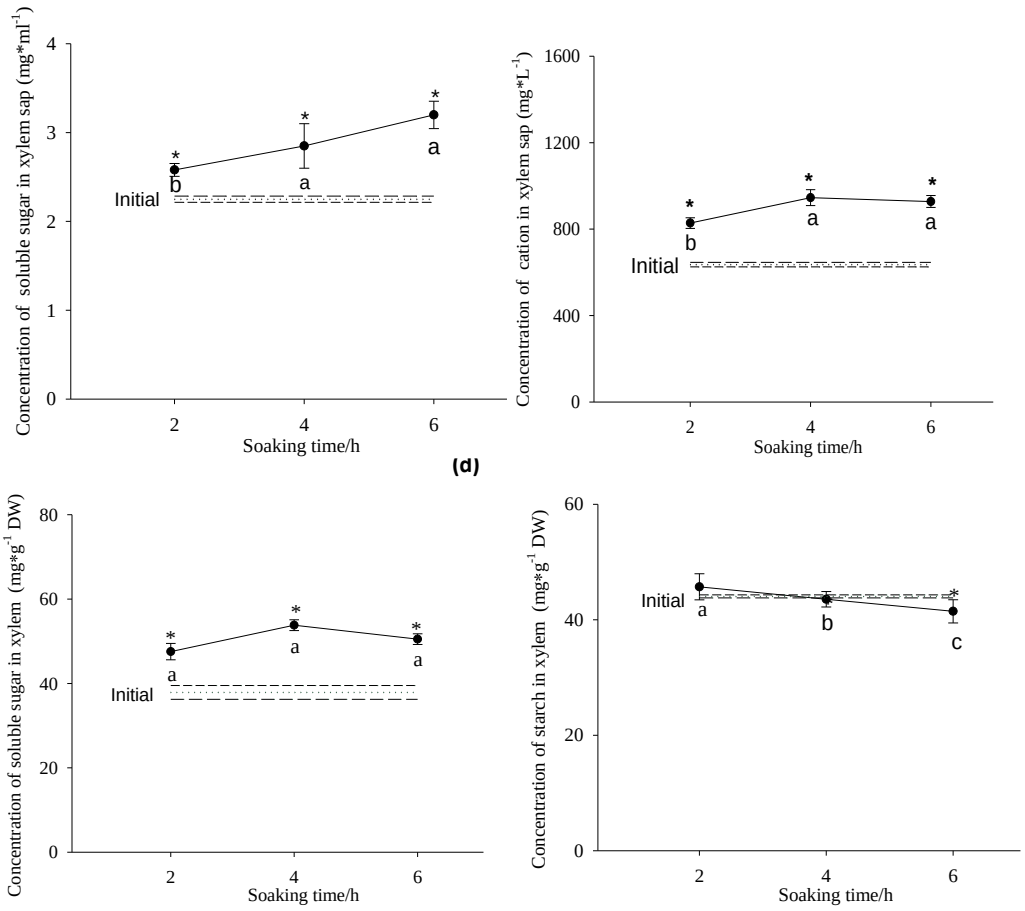

Fig. 2 Soluble sugar (a) and Cation (b) (the sum of Ca^{2+}, K^{+}, Mg^{2+} and Na^{+}) concentrations of xylem sap at different soaking times. Soluble sugar (c) and starch (d) concentrations of the xylem at different soaking times. Data in the figure are the mean ±SE (n=6). The dotted line represents the average initial value (before soaking in water); the dashed line indicates the initial mean ±SE (n=6). Different lowercase letters denote significant differences (P < 0.05) between soaking times; ∗ indicates significant differences (P < 0.05) between initial and after soaking values.

4. Discussion and Conclusions

The formation of xylem embolism is a non-biological process, which is influenced by the water column tension of the xylem and the physical and chemical properties of wood. However, the repair of embolism is a complex biological process hat requires not only the interaction of substance metabolism and energy metabolism but also sufficient water supply[9]. Studies show that plants can absorb water from fog, snow and rain to promote the hydraulic recovery of leaves, indicating that plants can directly absorb water from outside through non-root channels[10]. However, the role of bark in the hydraulic recovery of plants is only mentioned in some coniferous plants. We found that the weight of a branch of Salix increased by 134.37 mg after immersion in water for 24 hours, which is a result similar to that found in the research of Katz. This finding indicated that bark could absorb water and provide a water source for the repair of embolism, which showed that embolism could be repaired within 2 hours after refilling under moderate drought stress, but more than 20 hours were required after severe drought stress in Populus trichocarpaa[11]. Our study showed that the PLC decreased by 30.85, 21.24 and 34.37% after 2, 4 and 6 h of refilling, respectively, which indicated that exogenous water was transported radially to repair embolism in situ, but the time effect of such repair remains for further study.

As an important energy and carbon source in plants, soluble sugar is involved in many processes of plant life metabolism. Drought stress hinders the normal source-sink distribution of carbohydrates and reduces the total amount of carbon assimilation. Soluble sugar can maintain the specific structure and function of proteins by replacing the position of the water molecule and forming hydrogen bonds with the protein. The soluble sugar in the process of embolism repair may be transformed from starch in

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wood parenchyma cells. Starch hydrolysed into soluble sugar in parenchyma cells increases the swelling pressure of parenchyma cells and pushes water into the embolized vessels to promote the embolism repair under the effect of bulging pressure[12]. Buccifound that plants with high contents of soluble sugar in the petiole often had high water conductivity, and these soluble sugars were hydrolysed from starch in parenchyma cells of the xylem. Our study also found that in the process of refilling, the content of soluble sugar in the xylem sap increased, which is conducive to reducing the water potential of the embolism vessels and promotes more water to flow into the embolism vessels. However, how much soluble sugar comes from starch hydrolysis and what signal causes starch hydrolysis remains unclear.

Many studies report on the accumulation of intracellular inorganic ions as osmotic regulators under drought stress. The absorption of inorganic ions by plants is an active absorption process. The effects of inorganic ions on embolism repair primarily focus on the ions of K^+, Ca^{2+}, Na^+ and Mg^{2+}. The contents of K^+, Ca^{2+}, Na^+, and Mg^{2+} in leaves of Malus pumila and Juglans nigra increase under water stress. Tyree found that the contents of K^+ and Ca^{2+} in the unembolized branches of Laurus nobilis were higher than those in the embolized branches and that the content of K^+ in the branches increased significantly during the process of refilling. Our study found a similar result, and the contents of K^+, Ca^{2+} and Mg^{2+} in xylem sap increased during the process of refilling. K^+ is a primary substance of osmotic regulation in plants under drought stress and can regulate the accumulation of other osmotic regulating substances to a certain extent. Simultaneously, K^+ can mediate water to pass through pores. The content of Ca^{2+} in xylem sap increased significantly in the process of refilling, although some studies suggest that Ca^{2+} can increase plant susceptibility to embolism under drought conditions. We hypothesize that this finding may be observed because Ca^{2+} cannot improve the water conductivity of plants but can increase the effect of K^+ function.

In this study, we examined the role of bark water uptake in promoting xylem embolism repair in *Salix matsudana*. Therefore, we concluded that the driving force of embolism repair via bark water uptake was based on the osmotic gradient between the embolized vessels and vessel-associated cells, leading to water flow to the embolized vessels. The hydrolysis of starch into soluble sugar in xylem parenchyma cells and ion transport between cells were the primary factors affecting the composition of osmotic potential.

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