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(Article begins on next page)

Priming xylem for stress recovery depends on coordinated activity of sugar metabolic pathways and changes in xylem sap pH

Short running title: How does the biology of xylem apoplast impact the recovery from water stress in poplar?

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Abstract

In some plant species under low tension, the fast removal of embolism takes place during recovery from drought. However, the functional biology underlying this process still remains elusive.

We subjected poplar trees to drought stress followed by a period of recovery. Water potential, hydraulic conductivity, gas exchange, xylem sap pH and both carbohydrate content in xylem sap and woody stems were monitored in combination with an analysis of carbohydrate metabolism, enzyme activity and expression of candidate genes involved in sugar metabolic and transport pathways.

Drought resulted in an alteration of carbohydrate metabolism and a differential partitioning between starch and soluble sugars. Upon stress, an increase in the starch degradation rate and the overexpression of sugar symporter genes promoted the efflux of disaccharides (mostly maltose and sucrose) to the apoplast. In turn, the efflux activity of the sugar-proton co-transporters caused a drop in xylem pH. The newly acidic environment induced the activity of apoplastic invertases leading to the accumulation of monosaccharides in the apoplast thus providing the main osmoticum necessary for recovery.

During drought and recovery, a complex network of coordinated molecular and biochemical signals was activated at the interface between xylem and parenchyma cells that appeared to prime the xylem for hydraulic recovery.

Keyword index: apoplastic pH, disaccharides, drought, gene expression, monosaccharides, *Populus*, recovery, starch

Introduction

Embolism formation is a physical process influenced by a wide range of factors, including water tension, physical properties of the xylem, chemical properties of xylem sap, temperature and previous plant embolism history (Hacke *et al.*, 2001, Holbrook & Zwieniecki, 1999, Jensen *et al.*, 2016, Stiller & Sperry, 2002, Tyree & Zimmermann, 2002). Under drought stress or periods of high transpirational demand, increasing xylem tension increases the likelihood of embolism formation. Embolism accumulation progressively reduces stem water transport capacity, which can increase leaf water stress, forcing stomatal closure and reducing photosynthetic activity (Brodribb & Jordan, 2008). In the event of severe stress, when water loss by transpiration exceeds the transport capacity of xylem, runaway xylem embolism may occur leading to the complete cessation of water transport and, in the worst-case scenario, plant death (Sperry *et al.*, 1998). Therefore, it is conceivable that any strategy implemented by the plant to hinder and/or minimize the negative effects of embolism, including the restoration of hydraulic transport capacity upon stress relief, could be crucial for guaranteeing plant survival (Barigah *et al.*, 2013, Choat *et al.*, 2012, Tyree & Ewers, 1991).

Mounting experimental evidence proves suggests that during drought recovery a restoration of xylem functionality may occur in several plant species, even when the bulk of water in the xylem remains under low to moderate tension (Brodersen *et al.*, 2010, Nardini *et al.*, 2011, Secchi & Zwieniecki, 2011, Zwieniecki & Holbrook, 2009). Thus, recovery from embolism cannot happen spontaneously and requires the presence of living xylem cells in the proximity of empty vessels to facilitate the process and overcome existing energy gradients. Consequently, both the spatial arrangement and the amount of wood parenchyma could be crucial for successful embolism removal. The majority of living cells in the xylem are located in

parenchyma rays, which are often in direct contact with vessels. Parenchyma ray cells provide temporary storage for non-structural carbohydrates (NSC) in the form of sugar and starch (Salleo *et al.*, 2004, Spicer, 2014) and constitute a radial water redistribution pathway; both prerequisite functions that can promote the active repair of embolized conduits. *In vivo* observations show that during recovery, vessels fill up with water (Holbrook *et al.*, 2001, Scheenen *et al.*, 2007) initially derived from water droplets that preferentially form and grow on vessel walls that are in contact with living parenchyma cells (Tyree *et al.*, 1999, Brodersen *et al.*, 2010). However, while direct *in vivo* observations indicate parenchyma cells as important players in the hydraulic restoration process, the functional biology of this process remains unresolved.

According to current active embolism removal models, during water stress, starch in wood parenchyma cells is hydrolyzed to soluble sugars, which are transported along with ions to the apoplast (Secchi & Zwieniecki, 2012). Accumulation of osmotica decreases the apoplastic water potential allowing aquaporin-mediated water entry into the empty vessels upon relief from water stress. Once vessels have been refilled and become functional, sugars and ions are washed away with the transpiration stream (Brodersen & McElrone, 2013, Secchi & Zwieniecki, 2012, Secchi & Zwieniecki, 2016, Zwieniecki & Holbrook, 2009). These models are consistent with observations of NSC accumulation dynamics in parenchyma cells of drought-stressed plants. For instance, embolism presence has been shown to alter carbohydrate metabolism and the partitioning between starch and soluble sugars in xylem parenchyma (Salleo *et al.*, 2009, Secchi & Zwieniecki, 2011, Tomasella *et al.*, 2017) resulting in the accumulation of high levels of NSC content in trees subjected to short-term drought events (Trifilo *et al.*, 2017). Additionally, the ability to recover from embolism has been found to be species-specific and correlated with the concentration of soluble carbohydrates accumulated at the stem level (Savi *et al.*, 2016).

Shifts in carbohydrate metabolism during drought stress coincide with changes in apoplastic pH. Alkalization or acidification is one of the first chemical changes observed in the xylem sap of drought-exposed plants (Bahrn *et al.*, 2002, Sharp & Davies, 2009, Sobeih *et al.*, 2004) and can trigger the systemic activation of whole-plant water-stress responses (Schachtman & Goodger, 2008). A drop in xylem pH has been observed *in vivo* in poplar stems subjected to water stress (Secchi & Zwieniecki, 2012), and furthermore, *in vitro* analysis associated an acidic apoplastic pH to an increased accumulation of sugars in the xylem sap of poplar stems (Secchi & Zwieniecki, 2016). These observations suggest that the accumulation of sugars in the xylem apoplast is controlled by xylem pH, where a lower pH may induce apoplastic sucrose hydrolysis potentially via acidic invertase activity.

The directionality of sucrose symporters is determined by proton (pH) and sucrose concentration gradients across the plasma membrane (Carpaneto *et al.*, 2005). Thus, an increase of cellular sucrose content shifts the concentration gradient and triggers *de novo* efflux of sucrose to the apoplast. In functional vessels, such efflux would be unnoticeable as sugars are continually washed away. However, in embolized vessels, an increase in sucrose concentration, in combination with a lower pH in the xylem apoplast, would induce acidic invertase activity in the walls resulting in an accumulation of monosaccharides (mainly glucose and fructose). Sugar accumulation in the walls would significantly lower their osmotic potential and lay the basis for refilling upon relief from water stress (Secchi & Zwieniecki, 2012, Secchi & Zwieniecki, 2016). According to this model, changes in xylem apoplast chemistry should be coupled to membrane transport and cellular carbohydrate metabolism. We hypothesize that if the response of xylem parenchyma cells to severe stress is a coordinated biological process that results in the priming of xylem for hydraulic recovery, then concurrent changes in xylem pH, sugar composition and

concentration should be correlated with the expression of genes that affect carbohydrate metabolism and transport. In this study, we confirm that a lower xylem apoplastic pH is linked to carbohydrate accumulation in poplar, and we explore the changes in the transcriptional activity of candidate genes involved in sugar metabolism, transport and partitioning. The present work further supports the notion that severe water stress triggers a set of biological processes priming xylem for embolism removal upon watering (Secchi & Zwieniecki, 2014).

Materials and Methods

Plant material and experimental set up

One-year-old hybrid poplars (*Populus tremula* x *Populus alba* clone 717-1B4) were grown in a glasshouse under partially controlled climatic conditions. The average daily greenhouse temperature was $24.9 \pm 5.35^{\circ}\text{C}$ and relative humidity values ranged between 42.3 and 61.8 %. Maximum photosynthetic photon flux density (PPFD) ranged between 1330 and 1580 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 12-h-light/12-h-dark cycles were followed using halogen lamps, when necessary, to supplement light and guarantee a minimum PPFD of 500-600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Each plant grew in a 5 L pot filled with a substrate composed of sandy-loam soil/expanded clay/peat mixture (2:1:1 by weight). A total of 51 hybrid poplars with an average height of 180 ± 5 cm and stem diameter at the soil level of 11.6 ± 0.58 mm, were used in this study. The plants were divided into two groups: twenty-four poplars, belonging to group one, were used to estimate the level of embolism in response to water stress (PLC). Those plants were further divided into 3 subgroups: A) six plants watered daily (CTR), B) six plants severely water-stressed by withholding irrigation until the stem water potential (Ψ_{stem}) was below -2 MPa (STRESS), and C) twelve plants first stressed to below -2 MPa and then watered (PLC was

measured respectively after 1 and 7 days of recovery, REC1-7). The remaining twenty-seven poplars, belonging to the second group, were split into nine irrigated control trees (CTR) that were irrigated to water holding capacity daily, and 18 trees severely water stressed by withholding irrigation. Once severe water stress (SS) levels were reached in these 18 trees (day 0), half ($n = 9$) were sampled: xylem sap and tissues were collected and stored for further molecular and chemical analyses. The remaining half ($n = 9$) were watered (REC) during the morning of the same day (day 0) and allowed to fully recover over the period of seven days. After one week of stress relief (day 7), xylem sap and tissues were collected and stored for molecular and chemical analyses. Control plants ($n = 9$) were sampled throughout the experiment. Physiological parameters (Ψ_{stem} , stomatal conductance and photosynthesis), were monitored throughout the entire experiment (i.e. from the start of the stress treatment until full recovery of physiological functions) in droughted and control trees belonging to the second group. A detailed schematic representation of measurements/sampling timing is provided in Figure 1.

Measurements of stem hydraulic conductivity

Stem hydraulic conductivity was measured using a standard approach described previously by the authors (Secchi & Zwieniecki, 2012). In short, a long stem was cut under water and this initial cut was followed within a few minutes by cutting a set of three stem segments, each measuring approximately 4 cm. Segments were excised under water approximately 20–30 cm from the initial cut (a distance longer than 2 times the length of vessels in studied poplar). The initial hydraulic conductance (k_i) of each stem segment was measured gravimetrically by determination of the flow rate of filtered 10 mm KCl solution. A water source was located on a

balance (Sartorius $\pm$ 0.1 mg) and connected to the stem by a plastic tube. The stem was submerged in a water bath at a level approximately 10 cm below the water-level on the balance. After a steady flow rate was reached (a few minutes), the tube connecting the stem to the balance was closed, and a bypass was used to push water across the segment under approximately 2 bars of pressure for approximately 20 seconds to remove embolism. Stem conductance was then re-measured to find maximum conductance (k_{max}). The PLC was calculated as $PLC = 100 * (k_{max} - k_i) / k_{max}$.

Measurements of stem water potential and leaf gas exchange

For each plant, Ψ_{stem} was measured on equilibrated non-transpiring (bagged) leaves. First, mature leaves were covered with aluminum foil and placed in a humidified plastic bag for at least 30 minutes before excision. After excision, leaves were allowed to equilibrate for more than 20 minutes in dark conditions before being measured for water potential with a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). Physiological parameters were monitored daily (between 9:00 AM and 12:00 PM) for the duration of the experiments.

Stomatal conductance (g_s) and net photosynthesis (A_N) were measured on fully expanded leaves exposed to direct sunlight, using a portable infrared gas analyzer (ADC-LCPro+ system, The Analytical Development Company Ltd, Hoddesdon, UK). Measurements were performed using a 6.25 cm² leaf chamber equipped with artificial irradiation (1200 μ mol photon m⁻² s⁻¹), set with a chamber temperature of 25°C to avoid leaf overheating. CO₂ values were maintained at greenhouse conditions (400-450 ppm) for the duration of experimental trial.

Sap and stem sampling procedure

Xylem sap from functional vessels was collected from treated (SS-day 0 and REC-day 7, Fig. 1) and CTR plants, according to a previously described method (Secchi & Zwieniecki, 2012). Sap samples were kept at -20°C until analyses of ABA content, pH and soluble carbohydrate content were conducted. Using the same poplar stems taken for sap collection, wood samples were collected by peeling the bark and scraping the xylem using a sterile blade. Once collected, wood samples were immediately frozen in liquid nitrogen and kept at -80°C for further analysis (gene expression, starch and glucose content and enzymatic activity).

HPLC-MS/MS analysis of sap abscisic acid (ABA) content

ABA concentration was quantified following the method described by Siciliano *et al.* (2015), with minor modifications. After thawing, 50-100 µL of each biological replicate was centrifuged at 13000 g and 4 °C for 5 minutes. The obtained supernatant was filtered through a 0.2 µm syringe filter and collected in a 1 mL amber glass vial containing a glass insert (Supelco, Sigma-Aldrich) for small sample volumes and analyzed by HPLC-MS/MS. High Performance Liquid Chromatography was carried out using a 1260 Agilent Technologies (Waldbronn, Germany) system equipped with a binary pump and a vacuum degasser. Sample aliquots (20 µL) were injected on a Luna C18 (150 × 2 mm i.d., 3 µm Phenomenex, Torrance, CA), ABA was eluted in isocratic conditions of 65:35 (H₂O:CH₃CN v/v acidified with HCOOH 0.1%) under a flow of 200 µL min⁻¹ for 5 minutes. Using an electrospray (ESI) ion source operating in negative ion mode samples were introduced into a triple-quadrupole mass spectrometer (Varian 310-MS TQ Mass Spectrometer). Analyses were conducted in MRM mode using two transitions: 263>153 (CE 12V) for quantification, 263>219 (CE 12V) for monitoring, with 2 mbar of Argon (Ar) as

the collision gas. The external standard method was applied to quantify ABA concentration in target samples. A standard curve was generated using an original ABA standard (Sigma Aldrich, St Louis, MO, USA; purity 98.5 %), with concentrations ranging from 10 to 500 $\mu\text{g L}^{-1}$. Detection (LOD) and quantification (LOQ) limits were calculated based on the standard deviation of the response (σ) and slope of the calibration curve (S) ratio in accordance with the ICH Harmonized Tripartite Guideline expressed as: $\text{LOD}=3.3\sigma/\text{S}$; $\text{LOQ}=10\sigma/\text{S}$. Calculated final values were as follows: $\text{LOD} = 0.87 \text{ ng mL}^{-1}$; $\text{LOQ} = 2.90 \text{ ng mL}^{-1}$.

Analysis of starch and glucose concentrations in wood samples

Frozen wood samples were ground to a fine powder using a tissue lyser system (TissueLyser II, Qiagen), and starch content was quantified by enzymatic assay (STA-20 kit; Sigma-Aldrich), as detailed by (Secchi & Zwieniecki, 2011). Starch content was represented by the amount of released glucose, which was determined by colorimetric reaction using a glucose oxidase-mediated method in accordance with the manufacturer's instructions. Sample absorbance was read at 540 nm, and starch concentrations were calculated using the glucose standard curve as a reference and expressed as mg g^{-1} of fresh wood. Wood starch amounts were determined for all plants considered in the experiment.

For glucose measurements, $15 \pm 1 \text{ mg}$ sample of the same material was transferred into a 1.5 mL test tube. Each wood sample received 0.3 mL of 80 % ethanol solution before incubating twice at 80°C for 30 minutes. A third incubation was performed in 0.5 mL of assay buffer solution (50 mM Tris-HCl, pH 7.5). Glucose content was measured using the method described for sap.

In vitro enzymatic assays

For starch analysis, 50 µg of each sample were ground to a fine powder and suspended in 500 µL of acetate buffer. Samples were carefully mixed and centrifuged at 15000 rpm (Model 5415 C, Eppendorf, Milan, Italy) and the resulting supernatant was used to establish the basal concentration of glucose (the enzymatic method described above). Samples were then incubated at 55°C and 1 mM maltose was added to each. The glucose released by the hydrolysis of maltose due to native wood amiloglucosidases was measured after 5, 10, 30, 60 and 90 minutes, in order to define the linearity (inflexion point approx. at 60 minutes). *In vitro* amiloglucosidase activity was evaluated by subtracting the basal concentration of glucose from the glucose value measured after 30 minutes. Amiloglucosidase activity was expressed as hydrolyzed maltose ($\mu\text{mol min}^{-1} \text{g}^{-1}$ FW).

Measurements of pH and soluble carbohydrates in xylem sap

The pH measurements were taken on sap samples collected from CTR, SS and REC poplars using a micro pH electrode (PerpHect® ROSS®, Thermo Fischer Scientific, Waltham, MA USA). Concentrations of non-structural carbohydrates (NSC) in xylem sap were quantified following the anthrone-sulfuric acid assay described by (Leyva *et al.*, 2008); in short 50 µl of xylem sap were added to 150 µl of fresh anthrone reagent [0.1 g of anthrone (0.1%) in 100 mL of concentrated sulfuric acid (98%)], samples were mixed, kept for 10 minutes at 4 °C and then incubated for 20 minutes at 100 °C. After heating, samples were cooled for 20 minutes at room temperature before absorbance was read at 620 nm with a microplate reader (iMark Microplate Absorbance Reader, Bio-Rad). A glucose standard curve was used to compare the colorimetric response of the samples, and the NSC content was expressed as mol L^{-1} of glucose.

For glucose measurements, 5-20 μ L of sap were transferred to a cuvette with 2 mL (final volume) of assay buffer solution (50 mM Tris-HCl, pH 7.5, with 5 mM $MgCl_2$, 125 μ M $NADP^+$, and 1 mM $MgATP$ at 37°C) and placed in a spectrofluorimeter (LS50B Luminescence Spectrometer, Perkin-Elmer, MA, USA). The reaction was conducted by adding 2U of both glucose-6-phosphate dehydrogenase and hexokinase. When enzymatic kinetics reached a steady state, fructose was evaluated by adding 3U of phosphoglucose isomerase to convert hexokinase-produced fructose-6-phosphate to glucose-6-phosphate. Glucose concentration was then estimated from a calibration curve of known glucose amounts.

For sucrose analysis, 10-20 μ L of the supernatant were placed in a 1.5 mL test tube with 400 μ L of acetate buffer and 20 U of invertase enzyme to break down sucrose into fructose and glucose. Test tubes were then incubated at 55°C in a water bath for 30 minutes. The same procedure was followed for the digestion of starch breakdown soluble sugars (SSBP; maltose and its isoforms: isomaltose and maltodextrins), using 25 U of α -amylglucosidase per sample. To prevent further degradation, sap samples were boiled for 3 minutes. For the spectrofluorimetric analysis of the glucose produced by these reactions, 10-20 μ L of supernatant were transferred into a cuvette with 2 mL (final volume) of assay buffer following the same glucose quantification procedure described above. Finally, to quantify the amount of sucrose and SSBP, the baseline quantity of glucose was subtracted from quantities measured after the invertase and α -amylglucosidase reactions. All chemicals and enzymes used were from Sigma Aldrich (St Louis, MO, USA).

In addition, we compared NSC quantities in xylem sap using the anthrone-sulfuric acid assay in parallel with the enzymatic method (see above). A correlation between the two procedures (Fig. S1), confirmed the reliability of both techniques.

Real Time PCR experiments on wood samples

Expression changes of target transcripts were analyzed on wood samples collected from control CTR, SS, and REC poplars by quantitative real-time PCR (RT-qPCR). Frozen wood samples were first ground in sterile mortars using liquid nitrogen and further powdered by means of a tissue lyser system (TissueLyser II, Qiagen). Total RNA was extracted from 200 mg of powdered tissue following the method described by (Chang *et al.*, 1993) with slight modifications. Total RNA yield and purity were checked by spectrophotometry (Nanodrop ND-1000; Thermo Fisher Scientific), whereas RNA sample integrity was assessed by electrophoresis gel analysis. In order to avoid any risk of genomic DNA contamination, RNA samples were treated with DNase I (Invitrogen; Thermo Fisher Scientific) in accordance with the manufacturer's instructions. For each biological replicate, first-strand cDNA was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific), following the manufacturer's instructions. Real Time PCR reactions were carried out in a StepOnePlus™ RT-qPCR System (Applied Biosystems; Thermo Fisher Scientific), using the SYBR Green (Applied Biosystems; Thermo Fisher Scientific) method for quantifying amplification results. Thermal cycling conditions were as follows: an initial denaturation phase at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Expression levels of target transcripts were quantified after normalization to *Ubiquitin (PtUBI)* and *Actin (PtACT)* genes, both served as internal controls. Gene-specific primers used in Real Time PCR experiments are listed in Table S1. Real time PCR assays were carried out using three biological replicates per treatment-type and three technical replicates were run for each of the three biological replicates.

Statistical analyses

Significant differences among treatments were statistically analyzed by applying a one-way analysis of variance (ANOVA). Tukey's *HSD* post-hoc test was used for separating means when ANOVA results were significant ($P < 0.05$). Significant differences between pairwise comparisons were assessed by applying Student's *t*-test. The SPSS statistical software package (v24.0, SPSS Inc., Cary, NC, USA) was used to run the statistical analyses, and Sigma Plot software (Systat Software Inc., San Jose, USA) was used to create figures.

Results

Potted poplar trees reached a severe water stress level of -2.4 MPa (average stem water potential; Ψ_{stem}) after 11 days of imposed drought treatment (Fig. 2, day 0). The high level of drought imposed caused a severe loss of hydraulic conductivity ($80\% \pm 9.4$ of PLC, Fig. 3); and although the leaves lost their turgor, they did not shed, as shown in Fig. 3. Three days prior to a significant drop in stem water potential, stomatal conductance (g_s) and photosynthetic rate (A_N) were significantly lower in plants exposed to drought than in control trees (Fig. 2). At severe water stress levels stomata were fully closed (Fig. 2B; day 0). Half of the stressed plants (9 in total) were watered during the morning of day 0 and within several hours (day 1) Ψ_{stem} recovered close to control-plant levels (Fig. 2C). However, both g_s and A_N remained significantly lower and did not recover to pre-stress conditions until after three days of stress relief (Fig. 2A, B; day 3). Moreover, one day after stress relief, poplars did not recover from embolism formation (PLC = 64.2 ± 22.7), while a significant drop in the level of PLC, comparable to pre-stress level, was measured within seven days of watering (Fig. 3). Xylem sap ABA contents were significantly higher (up to 100 fold) in stressed plants (day 0) compared to control plants (Table 1). However,

after one week of rehydration ABA decreased to pre-stress values, reaching levels similar to those measured in control trees ($12.57 \pm 5.49 \mu\text{g L}^{-1}$ and $12.41 \pm 1.65 \mu\text{g L}^{-1}$, respectively), (Table 1).

Drought stress coincided with significant xylem sap acidification, from a pH of 6.28 ± 0.037 in control plants to 5.94 ± 0.042 in stressed plants (Table 1, Fig. 4A). Relief from water stress did not result in a pH recovery to pre-stress conditions even after seven days (Table 1, Fig. 4A). Under drought conditions, the acidification of xylem sap occurred in parallel with a significant increase in apoplastic soluble carbohydrate content (Fig. 4B). In SS poplars, NSC concentrations in xylem sap reached average values of $7.08 \text{ e}^{-03} \pm 1.31 \text{ e}^{-03} \text{ mol L}^{-1}$ (equivalent to 0.2 bar, a similar level as previously found in functional vessels of stressed poplar plants; Secchi & Zwieniecki, 2012), up to four times higher than in CTR plants (Table 2). The total amount of carbohydrates in the sap returned to pre-stress levels when water-stress was alleviated, with values overlapping those of irrigated poplars (Fig. 4B).

Plants subjected to water stress also exhibited a lower starch concentration in wood samples ($1.7 \pm 0.10 \text{ mg g}^{-1} \text{ FW}$) compared to CTR poplars ($13.2 \pm 0.47 \text{ mg g}^{-1} \text{ FW}$) (Table 3). The drop in concentration coincided with a transcriptional increase of beta-amylase-encoding genes (Fig. 5A). Drought stress was associated with an increase of transcript levels of *PtBMY1a*, *PtBMY1b* and to a lesser extent of *PtBMY3*. However, among the studied genes, only *PtBMY1a* was significantly over-expressed in the presence of stress. Interestingly, *PtBMY5* was the only gene over-expressed exclusively upon re-hydration and not under drought conditions (Fig. 5A). Since the final product of starch hydrolysis is maltose and its isoforms, the content of these products was quantified in the sap of SS poplars and was found to be five times higher than that measured in CTR sap (Table 2). Analysis of expression of a gene encoding a maltose transporter,

homologous to *AtMEX1*, also revealed an up-regulation in SS samples, which paralleled the increase in levels of sap maltose isoforms (Fig. 5B and Table 2). Since maltose derived from starch hydrolysis is further metabolized into glucose (Lu & Sharkey, 2006), we assessed both maltase (amyloglucosidase) activity and glucose content in woody tissues. We observed that maltase enzymatic activity had a tendency to increase in stressed tissues (Fig. 6), in agreement with the higher abundance of glucose found in SS wood samples (Table 3). In parallel to higher levels of maltose transporter transcripts, we found significant up-regulation of *PtSUT4*, a gene encoding a cellular membrane sucrose transporter (Fig. 5C). Transcriptional analysis, performed on wood samples, showed that *PtSUT4* was exclusively up-regulated in SS plants (Fig. 5C).

Analysis of basic sugar composition showed that sucrose concentrations in xylem sap slightly increased when water stress was induced, although not significantly, starting from $3.42 \times 10^{-4} \pm 4.37 \times 10^{-5} \text{ mol L}^{-1}$ and reaching values of $5.11 \times 10^{-4} \pm 1.35 \times 10^{-4} \text{ mol L}^{-1}$ when Ψ_{stem} was below -2 MPa (Table 3). In contrast, drought strongly increased glucose and fructose amounts in xylem sap, making them most abundant sugars in the apoplast of SS poplars, with up to 10 times the concentrations measured in CTR and REC samples (Table 3). Increased monosaccharide content was associated with higher levels of invertase expression; *PtCWINV1*, *PtCWINV2*, and *PtCWINV4* were previously found to be stem specific (Chen *et al.*, 2015). The *PtCWINV1* and *PtCWINV2* genes were expressed about ten and twenty times more than *PtCWINV4*, respectively and both were significantly up-regulated during stress and recovery. *PtCWINV4* transcription was exclusively induced in severely stressed samples characterized by an acidic apoplastic environment (Fig. 5D).

Discussion

Dynamics of physiological responses to severe drought followed by watering

Trees responded to increasing water stress with gradual decreases in net assimilation rate and stomatal conductance. More severe stress ($\psi_{\text{stem}} < -2$ MPa) strongly impacted the xylem hydraulic conductance inducing high levels of embolism formation (80% PLC), indicative of vulnerability to drought-induced cavitation in *P. tremula x alba* (Secchi & Zwieniecki, 2014). Although rehydration caused a fast recovery of stem water potential to pre-stress conditions, both A_N , g_s took three to seven days to fully recover, most likely the time needed to fully restore the stem's hydraulic capacity. This pattern of delayed recovery in stomatal conductance and photosynthetic processes, despite a quick recovery in xylem pressure, is consistent with previous results obtained in poplar (Secchi & Zwieniecki, 2014) and other woody plants, such as eucalyptus (Martorell *et al.*, 2014), grapevine (Chitarra *et al.*, 2014, Lovisolo *et al.*, 2008, Mitchell *et al.*, 2013), laurel (Trifilo *et al.*, 2017) and conifers (Brodribb & McAdam, 2013). Potted poplar trees grown in similar conditions were able to recover from embolism formation after water relief and under low or moderate tension; thus the results presented here are consistent with data of xylem hydraulic recovery measured respectively in *Populus trichocarpa* (Secchi & Zwieniecki, 2011), *P. nigra* (Secchi & Zwieniecki, 2012), and in the same clone used here (*Populus alba x tremula*; Secchi & Zwieniecki, 2014).

Besides hydraulic factors, delayed stomatal opening could be attributed to non-hydraulic chemical signals, such as abscisic acid (ABA) levels or xylem sap chemistry (Lovisolo *et al.*, 2002, Pantin *et al.*, 2013, Schachtman & Goodger, 2008). In this study, the decrease of xylem ABA sap content to pre-stress levels, which occurred over the period of seven days, coincided with the increase in stomatal opening. We can consider the observed delay in g_s recovery as a time buffer between the restoration of water potential and the onset of transpiration (Secchi &

Zwieniecki, 2014). In perennial plants, this buffer may provide the necessary time to repair the hydraulic transport capacity lost to embolism. Collectively, these results point to a dynamic regulation of stomatal and g_s recovery processes that may be influenced by a complex network of factors (i.e. changes in endogenous abscisic acid levels and/or efficiency of the photosynthetic system) beyond water supply through the xylem and stem water pressure.

Metabolism and partitioning between starch and soluble carbohydrates during drought and recovery

The ability of trees to repair xylem functionality after a short-term drought treatment is highly correlated with carbohydrate content in the stem (Trifilò *et al.*, 2017). Starch metabolism is considered to be at the forefront of plant response to embolism formation. Several studies have demonstrated that the presence of embolisms alters the metabolism and partitioning of starch and soluble carbohydrates, as well as their associated enzymatic activities and gene expression (Regier *et al.*, 2009, Salleo *et al.*, 2004, Secchi & Zwieniecki, 2011). Under stress conditions, both glucose content and maltase activity increased in stem parenchyma cells, thus confirming starch degradation as one of the initial plant responses to drought. The reduction in the starch content of stressed plants that we observed, is probably dependent on an enhanced degradation rate (Lu & Sharkey, 2006, Niittyla *et al.*, 2004, Weise *et al.*, 2005). Indeed, in SS samples MEX1, a maltose transporter-encoding gene homologous to *MEX1* in Arabidopsis known to direct maltose efflux to the cytoplasm (Niittyla *et al.*, 2004), was up-regulated. Up-regulation of MEX1, along with the concurrent increases in glucose content in parenchyma cells and maltose isoform levels in xylem sap, further supports the starch degradation process. Considering that, besides MEX, membrane SUT transporters also participate in the transport of maltose (Scofield

et al., 2007), while their gene expression is up-regulated under stress in poplar (Secchi *et al.*, 2011, Secchi & Zwieniecki, 2011), the increased cytoplasmic concentration of maltose isoforms may have led to efflux via SUT transporters and ultimately to the increase of maltose isoforms in xylem sap.

Moreover, analysis of beta-amylase expression, a crucial component for initiating the starch degradation process (Lu & Sharkey, 2006), further reinforces the idea that water stress triggers starch degradation. During the day, and/or when dehydration or osmotic stress occurs, BMY1 is the primary enzyme involved in starch breakdown (Valerio *et al.*, 2011, Zanella *et al.*, 2016). Here, *BMY1a* expression was up-regulated in SS wood samples, similarly, *BMY1a* was the only up-regulated β -amylase gene in the wood of *P. trichocarpa* under different osmotic stress treatments (Secchi & Zwieniecki, 2011). Interestingly, among the analyzed amylase genes, *BMY5* was over-expressed only upon rehydration. Although a clear understanding of the functional role of BMY5 is still missing, its up-regulation in REC samples, combined with a reduced starch content, is consistent with the over-expression of its homologous gene in *Arabidopsis* starch-less mutants (Monroe & Preiss, 1990), suggesting a potential involvement of BMY5 in the regulation of starch turnover during stress recovery.

Under drought stress, sugars in the xylem apoplast can accumulate either from the stored products of starch degradation in parenchyma cells (maltose and sucrose) or translocation from phloem to parenchyma rays (sucrose). Apoplastic accumulation can be either passive i.e. leaking of sugars across membranes due to large concentration differences between the apoplast and living cells, actively facilitated by membrane sugar transporters. The sucrose transporter *SUT4*, a protein homologous to the maize *SUT1*, is a phloem-localized bidirectional symporter which catalyzes both sucrose and proton transport depending on sucrose and pH gradients as well as

membrane potential (Carpaneto *et al.*, 2005, Carpaneto *et al.*, 2010). The role of *SUT4* in controlling sucrose transport and partitioning was explored in poplar and it was demonstrated that at the stem level, in addition to phloem cells, *SUT4* is also expressed in ray parenchyma cells, fibers and secondary xylem vessels (Payyavula *et al.*, 2011). We found that *SUT4* transcripts were more abundant in SS woody tissues. Therefore, the increased activity of this symporter can facilitate the efflux of both sucrose (and possibly maltose) and protons towards the apoplast, further evidenced by the increase in the total carbohydrate content of sap and by the observed acidification of apoplastic pH. A lower pH and an accumulation of disaccharides can halt the efflux of sugar via SUT transporters to the apoplast and consequently hinder the capacity to build sufficient levels of osmotica for refilling upon stress relief. However, a lower pH can also trigger the activity of apoplastic invertases, reducing the concentration of sucrose, thus maintaining the gradient prompting sucrose efflux.

Functional link between drought-induced acidification of xylem sap pH and sugar accumulation in the xylem apoplast

Alteration of sap pH is one of the first chemical changes that occurs within the xylem vessels of water stressed plants (Bahrun *et al.*, 2002, Sobeih *et al.*, 2004). Unlike herbaceous plants, drought-induced alkalization of apoplastic pH is not common in trees. Xylem sap acidification has been documented in a number of woody species (Sharp & Davies, 2009), most recently during the summer period in conifers at the alpine timberline (Losso *et al.*, 2017). Here, we show that: i) poplars exposed to severe water stress respond through acidification of the xylem apoplast; ii) a drop of pH under drought is accompanied by the accumulation of soluble carbohydrates; iii) glucose and fructose are the most abundant sugars in sap collected from

stressed poplars; iv) starch hydrolysis increases the content of soluble carbohydrates (i.e. maltose is also present in the sap). It was previously proposed that an acidic environment might cause the accumulation of monosaccharides in the xylem apoplast via sucrose hydrolysis mediated by acidic invertases (Secchi & Zwieniecki, 2016). Here, we further support that hypothesis through a gene expression analysis of members of the cell wall acidic invertases (CWINV). We profiled the expression of three genes, *CWINV1*, *CWINV2* and *CWINV4*, among the five members of the recently identified CWINV family in poplar (Chen *et al.*, 2015). *CWINV1*, *CWINV2* and *CWINV4* have been confirmed, by RNA-seq analyses on different tissues, to be expressed in the stem (Chen *et al.*, 2015). The results attest that all three genes were over-expressed during drought, accordingly elevated levels of glucose and fructose accumulated in the sap. Moreover, *CWINV 1* and 2 transcripts were overall more abundant than *CWINV4* (about ten times more for *CWINV1* and almost twenty times more for *CWINV2*), especially during recovery. This strongly implies that the biological control of physiological activity in response to stress is linked to an accumulation of monosaccharides in the xylem. This accumulation, in parallel with the reduction of disaccharide content, results in the steady efflux of disaccharides towards the apoplast – a transport sustained by low apoplastic sucrose concentrations and the fast buildup of monosaccharide osmotica. The fact that pH did not return to the pre-stress conditions within a few days, might reflect a lingering effect of stress or ‘memory of stress’ that would facilitate future responses in the case of consecutive droughts. Previous observations conducted *in vivo* in poplar stems, a drop in xylem apoplastic pH coincides with loss of stem hydraulic conductivity, and recovery of xylem pH induced by watering is delayed in comparison with recovery of stem water potential (Secchi & Zwieniecki, 2012).

Conclusion

In the present study, we strengthen and further support a previously proposed scenario wherein dynamic changes occur in the xylem sap at the onset of drought stress and recovery (Secchi & Zwieniecki, 2016). We characterize, from a molecular and biochemical perspective, the functional link between drought-induced xylem sap acidification and sugar accumulation, demonstrating that these synergistic events are crucial for triggering plant responses to water stress, including the eventual restoration of xylem functionality (Fig. 7). We thus unravel a complex network of molecular and biochemical signals activated at the interface between xylem and parenchyma cells during drought and its subsequent relief. Biochemical and molecular activity result in the upregulation of starch degradation processes under stress, followed by an overexpression of sugar symporters, which facilitate the efflux of disaccharides to the apoplast. Meanwhile, sugar symporter activity also mediates the reduction of xylem pH subsequently increasing acidic apoplastic invertases activity and stimulating the accumulation of apoplastic monosaccharides as the main osmoticum. In addition, water stress results in up-regulation of acidic invertase expression, increasing the potential for monosaccharide accumulation in the apoplast under stress. These coordinated events suggest ‘biological’ priming of xylem to expedite stress recovery during the rehydration period. Our work highlights the important role of carbohydrate reserve mobilization in sustaining the energetically demanding processes involved in plant stress responses.

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Table 1

Measurements of abscisic acid (ABA) and pH from xylem sap samples collected from well irrigated (CTR; day 0), severely stressed (SS; day 0) and recovered (REC; day 7) poplars. Average values of stem water potential (Ψ_{stem}) were also reported for each condition.

	CTR ($\Psi_{\text{stem}} = -0.44 \pm 0.015$ b)	SS ($\Psi_{\text{stem}} = -2.06 \pm 0.069$ a)	REC ($\Psi_{\text{stem}} = -0.36 \pm 0.038$ b)
ABA ($\mu\text{g L}^{-1}$)	12.41 ± 1.65 b	1189.16 ± 165.96 a	12.57 ± 5.49 b
pH	6.28 ± 0.037 a	5.94 ± 0.042 b	5.91 ± 0.053 b

Values are means $\pm$ SE (n = 9, each biological replicate represents a different plant). Different lower case letters following SE values indicate significant differences attested by Tukey's *HSD* test ($P < 0.05$).

Table 2

Concentrations of total carbohydrates, glucose, fructose, sucrose and starch breakdown soluble sugars (maltose and its isoforms: isomaltose and maltodextrins; SSBP) measured in xylem sap samples collected from well irrigated (CTR), severely stressed (SS, day 0) and recovered (REC, day 7) poplars. Analysis of total carbohydrate concentration was performed by anthrone method, while single sugars were measured by enzymatic assays. Average values of stem water potential (Ψ_{stem}) were also reported for each condition.

	CTR ($\Psi_{\text{stem}} = -0.44 \pm 0.015$ b)	SS ($\Psi_{\text{stem}} = -2.06 \pm 0.069$ a)	REC ($\Psi_{\text{stem}} = -0.36 \pm 0.038$ b)
Total carbohydrates (mol L ⁻¹)	$1.37 \text{ e}^{-03} \pm 1.54 \text{ e}^{-04}$ b	$7.08 \text{ e}^{-03} \pm 1.3 \text{ e}^{-03}$ a	$1.84 \text{ e}^{-03} \pm 8.32 \text{ e}^{-04}$ b
Glucose (mol L ⁻¹)	$1.23 \text{ e}^{-04} \pm 1.59 \text{ e}^{-05}$ b	$1.55 \text{ e}^{-03} \pm 3.96 \text{ e}^{-04}$ a	$8.78 \text{ e}^{-05} \pm 3.42 \text{ e}^{-05}$ b
Fructose (mol L ⁻¹)	$9.14 \text{ e}^{-05} \pm 1.86 \text{ e}^{-05}$ b	$1.69 \text{ e}^{-03} \pm 3.21 \text{ e}^{-04}$ a	$7.70 \text{ e}^{-05} \pm 3.85 \text{ e}^{-05}$ b
Sucrose (mol L ⁻¹)	$3.42 \text{ e}^{-04} \pm 4.37 \text{ e}^{-05}$ a	$5.11 \text{ e}^{-04} \pm 1.35 \text{ e}^{-04}$ a	$1.77 \text{ e}^{-04} \pm 1.19 \text{ e}^{-05}$ a
SSBP (mol L ⁻¹)	$1.15 \text{ e}^{-04} \pm 1.11 \text{ e}^{-05}$ b	$5.05 \text{ e}^{-04} \pm 1.71 \text{ e}^{-04}$ a	$7.34 \text{ e}^{-04} \pm 0.84 \text{ e}^{-05}$ b

Values are means $\pm$ SE (n = 9, each biological replicate represents a different plant). Different lower case letters following SE values indicate significant differences attested by Tukey's *HSD* test ($P < 0.05$).

Table 3

Measurements of starch and glucose content in stem wood samples collected from well irrigated (CTR), severely stressed (SS, day 0) and recovered (REC, day 7) poplars. Average values of stem water potential (Ψ_{stem}) were also reported for each condition.

	CTR ($\Psi_{\text{stem}} = -0.44 \pm 0.015$ b)	SS ($\Psi_{\text{stem}} = -2.06 \pm 0.069$ a)	REC ($\Psi_{\text{stem}} = -0.36 \pm 0.038$ b)
Starch (mg g ⁻¹ FW)	13.2 $\pm$ 0.47 a	1.7 $\pm$ 0.10 b	4.0 $\pm$ 0.18 b
Glucose (mg g ⁻¹ FW)	1.57 $\pm$ 0.20 a	2.04 $\pm$ 0.41 a	0.67 $\pm$ 0.26 b

Values are means $\pm$ SE (n = 9, each biological replicate represents a different plant). Different lower case letters following SE values indicate significant differences attested by Tukey's *HSD* test ($P < 0.05$).

Figure legends

Figure 1

Timing schematic representation of measurements/sampling over the duration of the experiment. Ψ_{stem} , stomatal conductance and photosynthesis were monitored throughout the entire experiment i.e. from the start of the stress treatment (day -11) until full recovery of physiological functions (day 7). PLC was measured on stressed and recovered poplars respectively at day 0, 1 and 7 (grey circles), while on control plants trough out the experiment duration (white circles). Chemical and molecular analyses were performed on sap and woody tissues collected at day 0 for stressed plants and at 7 days for recovered plants (grey circles). Sap and tissues from control plants were sampled trough out the experiment duration (white circles).

Figure 2

Measurements of (A) assimilation (A_N , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), (B) stomatal conductance (g_s , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and (C) xylem pressure (Ψ_{stem} , MPa) over the progression of drought stress (SS) and recovery (REC). Black dots indicate average values for each parameter, while grey and blue dots refer to single measurements of each parameter taken on SS and REC plants on each experimental day, respectively. The light blue rectangle represents the average value of each parameter measured on well irrigated (CTR) plants. Day -11 represents the beginning of the water stress treatment; Day 0 coincides with higher level of stress and with the moment of rehydration and Day 7 with full recovery. Asterisks denote significant differences between treated (SS or REC) and irrigated (CTR) plants on each day of measurements, tested using the Student's t test ($P < 0.05$). Bars represent SD ($n = 9$).

Figure 3

(A) Extent of embolism represented as percent of loss of conductivity (PLC) in stem of poplars
(B) Xylem pressure measured using balancing pressure method on transpiring leaves (Ψ_{stem} , MPa). White, grey, dark grey and black bars indicate average values measured in CTR, SS and 1 day and 7 days REC plants, respectively. Uppercase letters above bars denote significant differences ($P < 0.05$), tested using Tukey's *HSD* test. Error bars represent SE. (C) Severely stressed (day -3) and control poplar plants used in the experiment.

Figure 4

Changes in xylem sap pH (A) and total carbohydrate concentrations (B) as a function of increasing xylem pressure (Ψ_{stem} , MPa) measured in sap samples collected from severely stressed (SS), recovered (REC) and well irrigated (CTR) poplars.

Black, white and grey dots indicate average values measured in SS, CTR and REC plants, respectively. Smaller light grey dots refer to single measurements in SS and CTR or REC plants. Analysis of total carbohydrate concentrations was performed by anthrone method. Upper case letters above symbols denote significant differences, tested by Tukey's *HSD* test ($P < 0.05$). Bars represent SD ($n = 9$).

Figure 5

Expression changes of candidate genes involved in sugar metabolism and transport. RT-qPCR profiles refer to genes encoding (A) beta amylases (BMY), (B) maltose transporter (MEX1), (C) sucrose transporter (SUT4), and (D) cell wall acidic invertases (CWINV). Both Ubiquitin

(*PtUBI*) and Actin (*PtACT*) genes were used as endogenous controls for the normalization procedure of transcription levels. Uppercase letters above bars denote significant differences ($P < 0.05$), tested using Tukey's *HSD* test. Error bars represent SE ($n = 3$).

Figure 6

Measurement of maltase activity expressed as hydrolyzed maltose ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{FW}$) in wood samples obtained from well irrigated (CTR), severely stressed (SS) and recovered (REC) poplar stems. No significant differences were detected among treatments by the Tukey's *HSD* test ($P < 0.05$). Bars represent SE ($n = 9$).

Figure 7

Schematic of poplar stem biological responses analyzed in this study to severe water stress that result in priming of the stem for hydraulic recovery upon relief from stress.

Figure 1

760 **Figure 2**

Figure 3

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Figure 5

Figure 6

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