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ORIGINAL ARTICLE

B-type cyclin modulation in response to carbon balance in callus of *Populus alba*

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Abstract In plants, sucrose is the principal transported carbon compound. Sucrose and/or glucose and fructose are relevant in the regulation of meristematic cell proliferation. The aim was to define the sugar balance and the relationship between the osmoregulation processes and the cell division patterns in callus of *Populus alba* in response to different sucrose medium concentrations. Callus proliferation and metabolism were assessed through biometric measures, non-structural soluble carbohydrates quantification, osmotic potential determination, as well as by quantification of mRNA accumulation of B-type cyclin genes. Calli were cultured on proliferation medium without sucrose or supplemented with 10, 20 or 30 g l^{-1} of sucrose. A large amount of non-structural soluble carbohydrates was used to restore the osmotic balance between callus and medium; upon reaching the equilibrium, carbon was then used for cell division. The initial investment of carbon for osmoregulation processes can explain the differences in the lag phase duration in response to the decrease of medium sucrose concentration. However, calli cultured on medium added with 30 g l^{-1} of sucrose used carbon both to adjust their osmotic potential and to restore the growth whilst the calli on sucrose free medium entered in a quiescent state. The growth rates compared to the transcript accumulation trends suggested that a threshold effect, rather than a quantitative regulation model, governed the relation between *CycB* gene transcription and cell division. The findings showed that poplar calli used the carbon following a "hierarchical" model based on their physiological state and the sugar concentration available in the medium.

Keywords Poplar callus · Sucrose · Osmotic potential · Callus proliferation · B-type cyclins

Introduction

Soluble sugars play a central role in plant metabolism and sucrose is the main transportable carbohydrate in vivo as well as the most common carbon source used in plant tissue cultures. Carbohydrates can also act as signalling molecules, modulating gene expression and influencing different pathways (Eckstein et al. 2012). For example, sugars are reported as regulators of the gene expression of cell cycle checkpoint proteins (Rolland et al. 2002). Within in vitro systems, the type of exogenous sugars and their concentrations in the medium can have an important effect on callus growth (Jayaraman et al. 2014). Javed and Ikram (2008) showed that sucrose concentration had a significant effect on wheat callus growth; indeed, they found a decrease of fresh weight and an increment of dry weight of calli cultured with increasing sucrose concentration. Moreover, the increment of sucrose induced osmotic stress, as shown by the accumulation of proline; similar results were reported for rice callus (Khayri and Bahrani 2002). It is well documented that the whole plant responds to changes in water status by adjusting the osmotic level within the cells (Iannucci et al. 2002; Chaves et al. 2009). The osmotic adjustments can be achieved by accumulation

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of osmolytes, such as organic compounds, sugars and sugar alcohols, and they are put in place in order to maintaining an optimal cell turgor pressure (Valliyodan and Nguyen 2006). A high turgor pressure is required to support cell division and drive cell enlargement (Kirkan et al. 1972). Matsumoto et al. (1971) reported that high sugar concentration tended to lengthen the lag phase in a poplar cellular suspension, although subsequent growth rates were not significantly affected by the initial sucrose concentration. The lag phase duration depends mainly by the inoculum density/size while the growth rate by the cell state (Mustafa et al. 2011).

However, little information is available about the fate of sucrose added to the medium and the role of glucose and fructose, deriving from the sucrose hydrolysis, in the osmotic adjustments and the cell growth in in vitro systems (Khayri and Bahrani 2002; Asemota et al. 2007; Javed and Ikram 2008; Jayaraman et al. 2014). Moreover, information is lacking on how osmoregulation processes mediated by sugars could affect cell division in callus in particular of woody perennial species, such as poplar.

Sugar had a signalling role on cell cycle in model organisms such as yeast and *Arabidopsis* (De Veylder et al. 2003; Harashima et al. 2013). Cell cycle is an orderly and tightly controlled process, divided in 4 phases: G1, S (DNA replication), G2 and M (mitosis) (Howard and Pelc 1986; Nieuwland et al. 2009). Important checkpoints control the transition stages when cells move from the G1 into the S phase, and from the G2 into the M phase, primarily through the regulation of the activity of serine-threonine cyclin-dependent kinases (CDKs) (Fowler et al. 1998). In poplar, cyclins constitute a large multigenic superfamily of 45 genes that can be classified in seven groups: A, B, C, D, Q, T, and Z types; A, B, C, D, and T-cyclin subfamilies have orthologous groups in *Arabidopsis thaliana* while Q and Z types are exclusive of poplar (Dong et al. 2011). In plant, the G2-to-M transition is the crucial checkpoint for mitosis and is regulated by B-type cyclins, while D-type cyclins control the G1-to-S transition (Fowler et al. 1998). D-type cyclins are regulated by sucrose (Riou-Khamlichi et al. 2000; Stals and Inzè 2001; Nieuwland et al. 2009) while little information is available about sucrose regulation effect on mRNA transcriptional accumulation of B-type cyclin genes.

The purpose of this study was to define the sugar balance in the medium-callus system and how this could be related to the osmoregulation processes and cell division patterns. The main hypothesis was that sugars could be used both for mitotic activity signalling as well as for cellular osmoregulation, according to a carbon use "hierarchical" model based on the physiological state of the callus and the concentration of carbohydrates in the medium.

Since in vitro approaches are not influenced by environmental conditions or other variables, except the ones imposed (Demeter et al. 2014), calli derived from cambial meristematic cells of white poplar were used. European white poplar (*Populus alba* L.) is a species distributed along the river valleys of Europe, Asia and North Africa showing a wide genetic variability and the presence of ecotypes adapted to contrasting environments. It is a fast growing species with a good potential for biomass production in Mediterranean environment (Marron et al. 2010). For its commercial and ecological relevance, in recent decades significant efforts have been made to develop efficient protocols for in vitro regeneration (Confalonieri et al. 2003), genetic transformation for herbicide and insect resistance (Confalonieri et al. 2000; Delledonne et al. 2001) and germplasm preservation (Lambardi et al. 2000). Furthermore, this species has been used as a model system to unravel the molecular mechanisms involved in drought response (Berta et al. 2010; Luisi et al. 2014) and salt adaptation (Beritognolo et al. 2011).

Callus proliferation and cell metabolism were assessed through biometric measures, non-structural soluble carbohydrates (NSC) quantification, osmotic potential ($\Psi\pi$) determination, as well as by quantification of mRNA accumulation of B-type cyclin genes for their role in the control of mitotic activity.

Materials and methods

Collection of *P. alba* samples and in vitro culture initiation

Twigs, 20–30 cm long, were collected in January from a mature tree of *Populus alba* (clone Marte) growing in the nursery of the CNR-IVALSA Institute in Sesto Fiorentino, Florence, Italy (43°48′N, 11°11′E). The twigs were immediately placed in distilled water, maintained at 22 °C and 100 % humidity, to force the onset of cambium activity.

The cambium activity was monitored by optical microscope on 12 µm thick twig sections. After the onset of cambium activity, the twigs were cut into 5 cm segments, washed in running tap water for 30 min. The surface of the segments was disinfected with 70 % ethanol for 1 min, followed by 2 % sodium hypochlorite for 20 min and rinsed 3 times with sterilized distilled water.

The cambial zone was aseptically peeled off from the inner side of the bark under laminar flow. Thin cambium slices (less of 1 cm long) were laid on Petri dishes containing standard Proliferation Medium (PM) composed of MS (Murashige and Skoog 1962) medium supplemented with 4.4 µM 6-benzyladenine (BA), 5.4 µM α-naphthalene

acetic acid (NAA), 30 g l^{-1} of sucrose, 0.6 % plant-agar (Duchefa, The Netherlands). The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. To stimulate callus induction the explants were incubated at 23 ± 1 °C in the dark.

After 10 days on PM, callus formation was evident on primary explants and, after 30–40 days, dedifferentiated cells covered all the explant. The new callus portions were gently separated from the primary explants, transferred onto Petri dishes containing fresh standard PM and sub-cultured two times for 4 weeks. After this period, the calli were soft, compact and fast growing.

Experimental trials

Only fresh, viable and proliferating calli were used in the trial. In order to avoid the use of dedifferentiated cells with asynchronous growth, the calli were cultured for 14 days more on standard PM before the trial started. At this time the calli showed an exponential growth as also observed in precedent experiments (data not shown). The proliferating calli were cut into small pieces (0.8–1 g in weight) and cultured individually on Sucrose Free (SF) PM or supplemented with 10, 20 or 30 g l^{-1} of sucrose (10, 20, 30 PM). All cultures were incubated at 23 ± 1 °C in the dark for 4 weeks (Supplementary Fig. S1).

Biometric analysis

Every week the fresh (FW) and the dry weights (DW) of the calli were determined on five biological replicates for each PM. For the FW, the calli were weighted immediately after removal from PM (blotting them to remove the excess of water). The DW was measured using the Moisture Analyzer HG63 (Mettler Toledo) with the temperature set at 200 °C.

The Water Content (WC) was calculated as follow:

$$WC = (FW - DW)/DW$$

The Relative Growth Rate (RGR) was calculated on the FW basis as follow:

$$RGR = [\ln(FW_1) - \ln(FW_0)]/(t_1 - t_0)$$

The sampling interval ($t_1 - t_0$) was one week (days 0–7, days 8–14, days 15–21, days 22–28).

Quantification of non-structural soluble carbohydrates (NSC)

Every week NSC were extracted from the calli and the media. For each sampling day, three Petri dishes were randomly selected for each different PM. The calli were removed from PM and frozen at −80 °C. At the same time, PM samples (1 × 1 × 0.2 cm) were collected and NSC were immediately extracted. For the extraction, frozen calli or PM were placed in 2 ml Polypropylene Spin-X centrifuge tube (Costar®, Corning Inc., Corning, NY, USA) equipped with 0.22 µm cellulose acetate filter and centrifuged at 10,000*g* at 5 °C for 10 min. The filtrate (cell content or PM) was recovered and used for NSC analysis. The NSC content was determined as described in Giovannelli et al. (2011). Sugar separation was performed by high-performance liquid chromatography using a SHODEX SUGAR Series SC 1011 8 × 300 mm column (Showa Denko, Germany), preceded by a pre-column Guard Pak Insert Sugar Pak II (Waters). The mobile phase was water, Milli Q grade, at 0.5 ml min^{-1}. NSC identification was verified using authentic standard carbohydrates (Sigma-Aldrich).

Osmometer analysis

Analysis was carried out on the filtrate deriving from callus and medium samples used for NSC determination. Each sample (25 µl) was analysed by a freezing point osmometer (Osmomat 030 Gonotec, Germany). The Ψπ of the solution was calculated for 25 °C based on the Van't Hoff relation:

$$\Psi_\pi(\text{MPa}) = 0.002437\ (\text{m}^3\text{MPa mol}^{-1}) \times \text{osmolarity}\ (\text{mol m}^{-3}).$$

Primer design, RNA isolation and RT-PCR analysis

In order to design primers for RT-PCR analyses, the nucleotide coding sequences (CDS) of *Populus trichocarpa* cyclins were retrieved from the Phytozome database (http://www.phytozome.net/). The black cottonwood cyclin B1 NCBI reference sequence XM_002313980.1 was used as seed for a Blastn analysis on the *P. trichocarpa* genome database. The sequences showing significant hits were downloaded from the Phytozome database, aligned with Muscle (Edgar 2004) (multi-alignments and primer position maps are reported in Supplementary Figs. S2–S8), trimmed to eliminate poor aligned regions and used to build a Neighbor Joining tree (1000 bootstrap) with Mega 5 (Tamura et al. 2011) for the cyclin belonging to the B, A, and D-type. The dendrogram (Supplementary Fig. S9) was used to clarify phylogenetic relationships among the B-type cyclin genes to guide primer design. Primers were designed to amplify simultaneously (following gene naming reported in Dong et al. 2011) the pairs: *CycB1.1* and *CycB1.2* (cluster A), *CycB1.3* and *CycB1.4* (cluster B), *CycB2.3* and *CycB2.4* (cluster C). Due to the sequence divergence that did not allow designing a single primer

pair, primers were designed to amplify separately *CycB2.1* and *CycB2.2*. Table 1 reports the principal features of the primers used. The *CycB2.2* gene transcript accumulation was not analysed because it was not possible to obtain good RT PCR amplification.

For extracting RNA, calli were crushed with liquid nitrogen in sterile mortars. The samples were always stored at −80 °C before RNA extraction. Total RNA was extracted on two biological replicates for each treatment, according to Chang et al. (1993), modified by removing spermidine and adding β-mercaptoethanol into the extraction buffer. RNA integrity was checked by denaturing agarose gel electrophoresis and quantified by determining the sample absorbance at 260/280 nm using the Eppendorf Biophotometer (average concentration of total RNA was 1000 ng μl^{-1} and average 260/280 ratio > 1). An amount of 3.0 µg of total RNA was treated with *DNAse I* to remove DNA contamination using the "*DNAse* I Amplification Grade" kit (Invitrogen) according to the manufacturer's protocol. After *DNAse I* treatment, total RNA was precipitated with LiCl and re-suspended in 50 µl of DEPC water. The re-suspended RNA was retro-transcribed in first-strand complementary DNA (cDNA) using the reverse-transcription polymerase chain reaction system "SuperScript VILO™ MasterMix" (Invitrogen) according to the manufacturer's protocol. The reaction was performed using random primers in 25 µl of final volume. cDNA was used in RT-PCRs to determine *CycB1.1-2-3-4* and *CycB2.1-3-4* mRNA accumulations, compared with *Act-2* and *Ef-1*, using the ΔΔCq method (Pfaffl 2001) taking into account for differences in gene specific amplification efficiencies. Primers for reference genes *Act-2* and *Ef-1* were obtained from Brunner et al. (2004) and Pallara et al. (2012), respectively. Target and reference gene-specific amplification efficiencies were calculated using the quantification cycles (Cqs) of a 4-point dilution set (1:4, 1:8, 1:16, and 1:32) of standard samples in duplicate reactions. Efficiencies were calculated inside qBase (Hellemans et al. 2007) taking into account Cqs variations registered for the standard samples for each gene and dilution. RT-PCRs were performed in duplicate reactions for each sample and the results were discarded in the presence of a difference in Cq values for replicates higher than 0.5. In the same experiment, the highest possible number of samples for the same gene was amplified in order to facilitate inter-sample comparisons. In each run, and for each gene, No Template Control (NTC) was amplified, resulting in Cqs always higher than 38. RT PCRs were performed with an annealing temperature of 60 °C for all the genes using the "Express SYBR greener qPCR Supermix Universal" kit (Invitrogen) with 10 µM of each primer and 2 µl of a 1:8 dilution of template cDNA in a final volume of 20 µl. The Cqs obtained for the samples were always comprised in the lower and upper Cqs limits of the standard curves. The complete qPCR protocol was as follows: 95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s for 40 amplification cycles. At the end of the amplification cycles, the melting curve for each amplicon was calculated in order to check for the presence of secondary amplification products.

Statistical analysis

Data were checked for normal distribution (Kolmogorov–Smirnov D test). The effect of sucrose concentration within the calli and the PM during the trial was tested with a two-way analysis of variance (ANOVA) to assess if the differences found between the samples were statistically significant ($p \leq 0.05$). The data that did not pass the Kolmogorov–Smirnov D test were opportunely transformed before the ANOVA. The Pearson correlation coefficient, and its level of significance, was used to quantify the correlation between NSC contents in the calli and in the PM and between sugars and Ψπ. All the analyses were performed with the STATISTICA 10 software (Statsoft, Poland).

Table 1 Primer sequences

Primer name	Sequence (5′–3′)	Target sequence (s)	Annealing temperature (°C)	Amplicon size (bp)
CycB1.1-2 F	GAGATCGATCCTCGTAGATTGG	Potri.009G066600.1	60.1	199
CycB1.1-2 R	TGGTGCCCATATTTCTTCGT	Potri.001G272000.1	60.3	
CycB1.3-4 F	GAGCCATTCTTGTGGATTGG	Potri.006G035200.1	60.5	224
CycB1.3-4 R	AGCTCTGTCTGAAACGCACA	Potri.016G033000.1	59.8	
CycB2.1 F	AGAGGAGATGGCAGCAGAAA	Potri.009G165800.1	60.1	225
CycB2.1 R	CTTTCAAATCGCCACAGTCA		59.8	
CycB2.2 F	CCGTGGTGTTTGTGAAGAAC	Potri.005G100000.1	59.0	173
CycB2.2 R	CGGAAGATGCCTTGTTTTCT		59.3	
CycB2.3-4 F	GCTAACAAGCAGCAGCAACA	Potri.005G251400.1	60.3	246
CycB2.3-4 R	GTCACAGCCGTCTATGTCCA	Potri.002G010000.1	59.7	

Results

Growth and callus water content

The data showed that sucrose concentration into the PM determined the fate and the kinetic of the callus growth (Fig. 1). Depending on sucrose concentration in the PM, four different growth kinetics were observed: (a) on SF PM the calli ceased to proliferate, entering in a quiescent state and remaining in this condition until the end of the experiment; (b) on 10 PM the calli showed a 2 weeks long lag phase, then their proliferating capacity increased gradually; (c) on 20 PM the lag phase was reduced to 1 week: then the RGR increased considerably (higher than 40 mg FW day^{-1}) from the 2nd week of culture; (d) on 30 PM the calli maintained the proliferation capacity after subculturing them in the fresh PM, even if a low RGR (<10 mg FW day^{-1}) was recorded at the end of the 1st week. These results showed that high sucrose concentrations had a stimulating effect on the recovery of cell division after subculturing. Callus WC ranged between 7 and 12 g H_2O g^{-1} DW throughout the trial (Fig. 2). Two-way ANOVA showed that callus WC did not change significantly during the experiment and it was not affected by sucrose concentration added to PM ($p = 0.2$).

Kinetics of sucrose hydrolysis within the PM

Sucrose concentration within PM was not affected by autoclaving. After autoclaving, more than 90 % of initial sucrose was found again within PM (Fig. 3) and its concentration did not change over time (data not shown). On the contrary, when calli were placed on PM sucrose concentration rapidly decreased with a defined pattern. Regardless of the initial concentration, sucrose was detected only in traces at the end of trial.

Two-way ANOVA, reported in Supplementary Table S1, showed that the hexose concentrations were significantly affected by initial sucrose content in PM and by the time. In particularly, more than 50 % of sucrose in all sucrose enriched PM was hydrolysed in glucose and fructose after 1 week of culture (Fig. 3). During subculture time course, glucose content decreased while fructose had an increase until the 3rd week of culture and after decreased. At the 4th week of the experiment in all PM the sum of hexoses was over 96 % of NSC concentration and fructose was the highest NSC detectable (>75 mM in the calli on 30 PM).

Kinetics of NSC within the calli

At the beginning of the trial (day 0), the callus NSC composition was 6.05 mM of sucrose, 23.98 mM of glucose and 46.72 mM of fructose. Fructose and glucose were the highest NSC found in the calli after 1 week of culture (Fig. 4). Irrespective of sucrose concentration, they represented more than 95 % of the total NSC in the calli on 30 and 20 PM and more than 86 % on 10 PM. Between the 2st and the 3rd week of culture, glucose decreased (60 % in the calli on 20 and 30 PM) more than fructose (on average 40 % in the calli on 30 and 20 PM), showing that at this stage the calli used it to support growth and cell metabolism. Contrary to hexoses, sucrose concentration within the calli did not significantly change during the trial ($p = 0.10$, Supplementary Table S1), except in the calli on 10 PM where the disaccharide disappeared between the 3rd and the 4th week. On SF PM, the NSC within the calli were rapidly metabolized in the 1st week of culture and they were not detectable after this time.

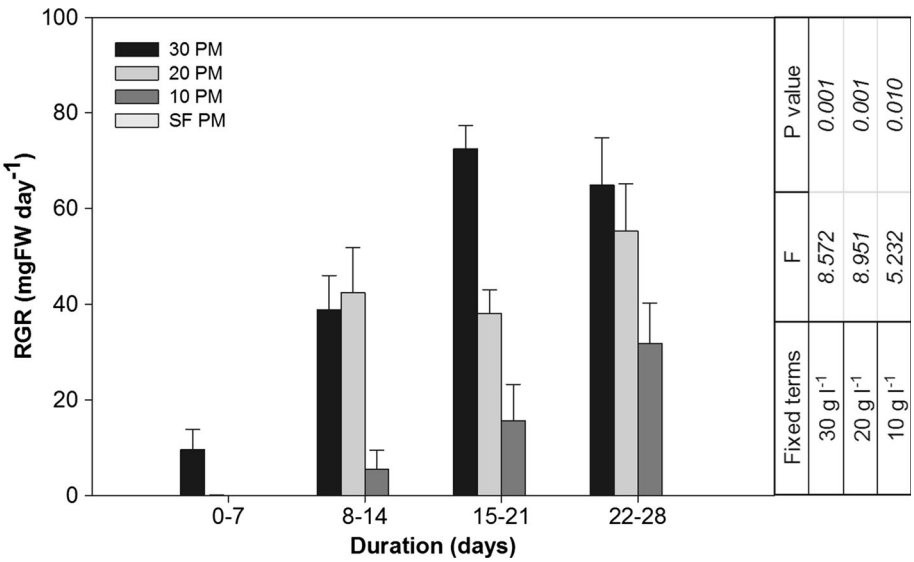

Fig. 1 Daily RGR (mg FW day^{-1}) of callus cultured on 30, 20, 10, and SF PM during the time course experiment; on the right the results of two-way ANOVA

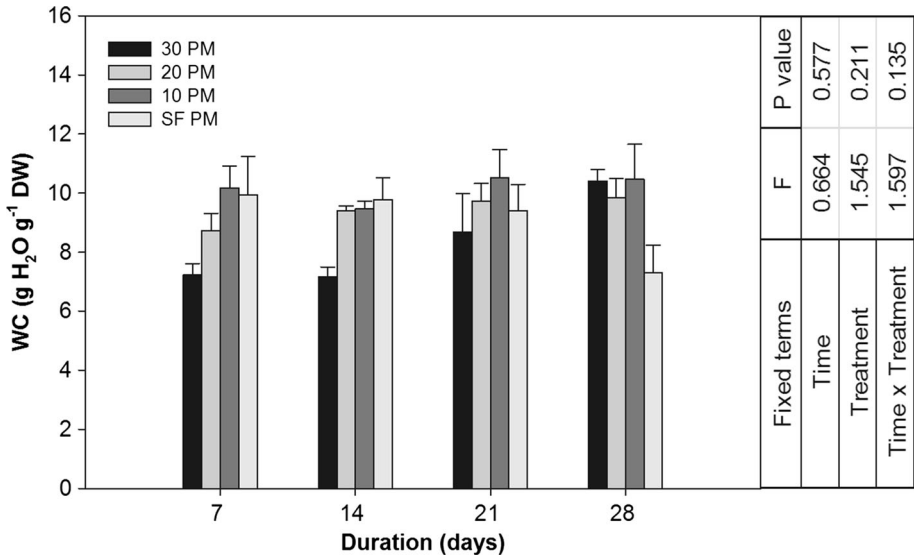

Fig. 2 Callus WC on 30, 20, 10, and SF PM during the time course experiment; on the right the results of two-way ANOVA

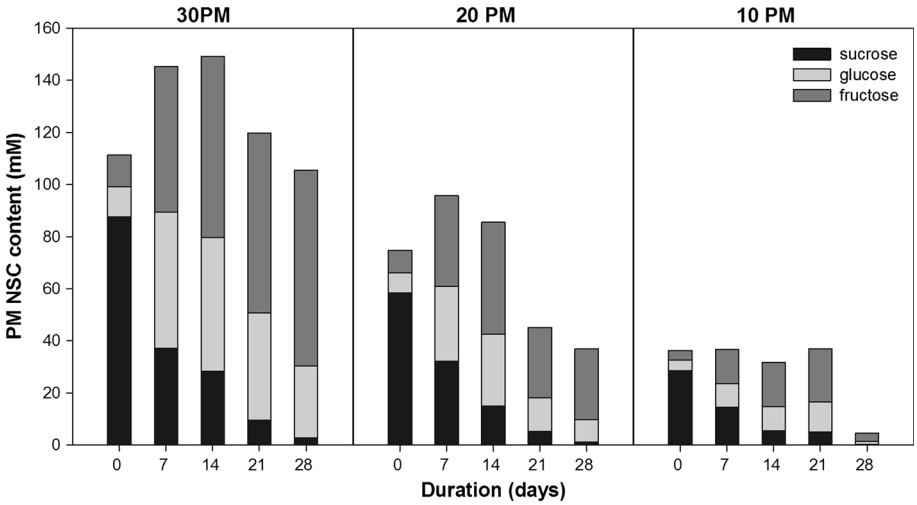

Fig. 3 NSC concentration in PM; at the beginning of the experiment NSC content was measured before the callus transfer to PM

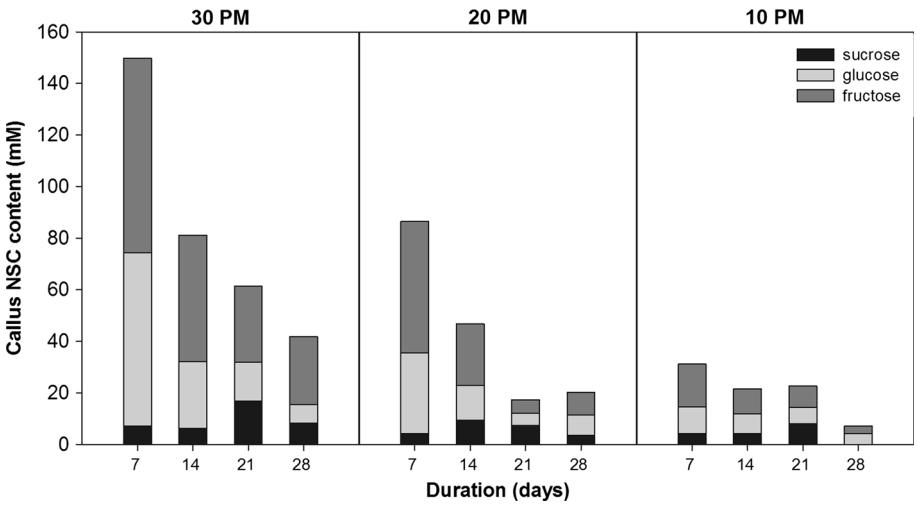

Fig. 4 NSC concentration in the calli cultured on 30, 20, 10 PM during the time course of the experiment. NSC concentration on SF PM medium was undetectable

NSC balance within the PM-callus system

Irrespective of initial sucrose concentration, the NSC content of all PM was positively correlated with that measured in the calli ($R = 0.933$, $p < 0.0001$) showing that the carbon fluxed from PM to the calli was transformed in cellular structures and energy to support growth or stored as starch. Consequently, the rate of NSC decrease in the calli was significantly affected by initial sucrose concentration in PM.

Changes in the osmotic potential

When callus was transferred to the proliferation medium at the beginning of the experiment, its $\Psi\pi$ was on average -0.75 ± 0.10 MPa, whilst the $\Psi\pi_{medium}$ changed according to the added sucrose concentration (-0.70 ± 0.02 MPa, -0.57 ± 0.03 MPa, -0.46 ± 0.02 MPa, -0.36 ± 0.02 MPa in 30, 20, 10, and SF PM, respectively). At this time the differences in $\Psi\pi$ between the proliferating callus and the fresh PM ($|\Psi\pi_{callus\text{-}medium}|$) were significantly affected by the initial sucrose concentration in PM ($p < 0.0001$). Consequently callus transferred on SF or on 10 PM had a higher $|\Psi\pi_{callus\text{-}medium}|$ (0.294 and 0.389 MPa, respectively) than those placed on 20 and 30 PM (Fig. 5). The $\Psi\pi_{callus}$ and $\Psi\pi_{medium}$ patterns during the trials are reported in Supplementary Fig. S10 and Fig. S11.

After only 1 week on PM, the $|\Psi\pi_{callus\text{-}medium}|$ ranged from 0.18 to 0.25 MPa, showing that active osmotic adjustments took place within the calli. $|\Psi\pi_{callus\text{-}medium}|$ was significantly affected by the initial sucrose concentration in PM ($p < 0.0001$). During the subculture, $\Psi\pi_{callus}$ was always lower than $\Psi\pi_{medium}$ (-0.2 MPa on average) in all PM showing that an osmotic equilibrium was reached within the callus-medium system. Since sucrose was rapidly hydrolysed by calli, the osmotic adjustments were mainly driven by glucose and fructose. In fact, a positive correlation was found between glucose and fructose concentrations in PM and $\Psi\pi_{medium}$ ($R = 0.844$ and 0.763 respectively, $p < 0.0001$).

Transcript accumulation of mRNA of B-type cyclin genes

Phylogenetic analysis showed that poplar B-type cyclin subfamily was composed by 9 genes, further divided in 3 phylogenetic groups: *CycB1* (4 genes), *CycB2* (4 genes), and *CycB3* (1 gene). The mRNA accumulation of the analysed genes was cross-calibrated to evaluate the different transcription of the isoforms showing that they had different activity. During the experiment time course the two subfamilies of cyclins, B1 and B2, had a similar transcript accumulation pattern in the calli on different PM. The mRNA accumulation of B1 and B2 cyclin isoforms (Fig. 6 and Supplementary Fig. S13) slightly decreased during the experiment time course and the differences found were determined by the different sucrose contents in PM at the beginning of the experiment. mRNA transcriptional accumulation of B-type cyclin genes was higher in the calli on 20 PM, than on 30 PM, except at the 2nd week of culture when *CycB* genes were more transcribed in the calli on 10 PM. The calli on SF PM did not have an accumulation of mRNA transcribed from *CycB* genes after the 3rd week of culture, but they had an accumulation of mRNA transcribed from housekeeping genes. Two-way ANOVA, reported in Supplementary Table S2, showed that the differences in mRNA accumulation were statistically significant for time, treatment and the interaction between the two variables.

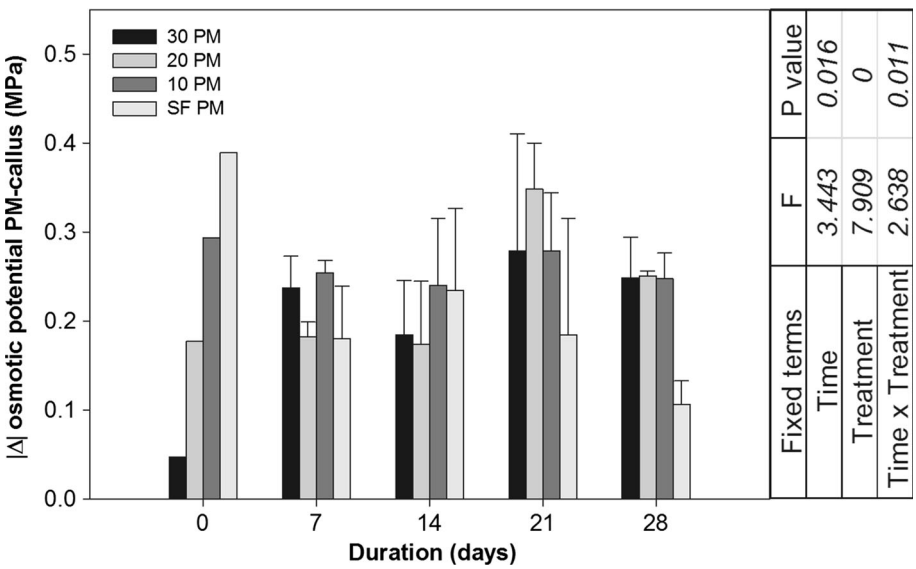

Fig. 5 Differences between the osmotic potential of the callus and the PM ($|\Psi\pi_{callus\text{-}medium}|$) during the time course of the experiment; on the right the results of two-way ANOVA

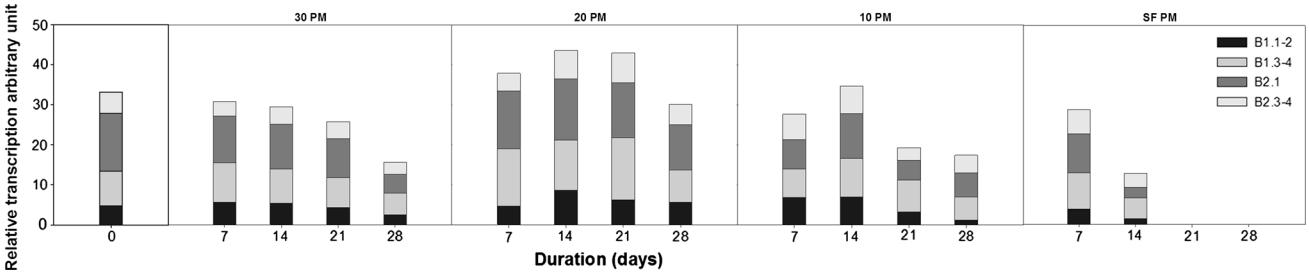

Fig. 6 Relative mRNA accumulation of B1 and B2 cyclin genes; *CycB1.1-2* are reported in *black*, *CycB1.3-4* in *medium gray*, *CycB2.1* in *dark gray* and *CycB2.3-4* in *light gray*

Transcript accumulation of mRNA from *CycB1.1* and *CycB1.2* genes slightly increased until the 2nd week of culture in the calli on sucrose enriched PM and then decreased toward the end of the trial. During the experiment time course, the transcript accumulation pattern of these isoforms followed a similar trend in the different PM: they were transcribed in the calli on 20 PM more than on 30 and on 10 PM and less transcribed on SF PM. Transcript accumulation of mRNA of *CycB1.3* and *CycB1.4* genes showed a trend similar to those shown by *CycB1.1* and *CycB1.2,* except after the 1 week of culture. Isoforms 3 and 4 of *CycB1* were transcribed more than isoforms 1 and 2 (about 50 % of relative transcription). Transcript accumulation of *CycB2.1* gene was abundant in the calli on 20 PM more than on 10 and on 30 PM and it was always less abundant on SF PM. *CycB2.3* and *CycB2.4* had a similar time course trend of *CycB2.*1, but in general they were less transcribed than the latter (about −50 % of relative transcription). No isoform was transcribed after 3 weeks of culture in the calli on SF PM.

Discussion

The findings showed that dedifferentiated cells of poplar used the carbon following a "hierarchical" model based on their physiological state and the sugar concentration available in PM. After subculturing, a large amount of NSC was used to restore the osmotic balance between callus and PM and upon reaching the equilibrium, carbon was then used for cell growth. The initial investment of carbon toward osmoregulation processes can explain the differences in the duration of the lag phase at the beginning of the subculture in response to the decrease of sucrose concentration in the PM.

As expected, the presence of sucrose in the PM stimulated callus proliferation. The highest growth rate was recorded in the calli on 30 PM (with a peak of 72 mg FW day^{-1}) whilst the calli on SF PM did not grow. It is well documented that 30 g l^{-1} is the standard concentration of sucrose used in in vitro systems (Tisserat 1982; Nuutila et al. 1991; Asemota et al. 2007). Few authors showed that the callus weight increased with the increment of sucrose concentration in the medium until 30 g l^{-1}; if sucrose concentration had a further increase the callus weight decreased (Tisserat 1982; Asemota et al. 2007). In contrast, a recent study on *Aquilaria malaccensis* showed that the optimal sucrose concentration for the callus growth was 15 g l^{-1}, whilst the optimal glucose concentration was 25 g l^{-1} (Jayaraman et al. 2014). However, in the present study was also found that the increase of sucrose concentration in PM induced a reduction of the lag phase after subculturing. This result was evident when proliferating callus was placed on 30 PM. Under this condition callus continued to proliferate during the 1st week of culture. On the contrary, the lag phase increased in response to the decreasing of sucrose in PM. These findings were confirmed by the quiescent state of the calli when they were subcultured on SF PM. Matsumoto et al. (1971) reported similar results in poplar suspension culture: high sugar concentration tended to lengthen the lag period in the growth curve, although there was no difference among the growth rate in the exponential phase for each concentration.

The different sucrose concentration in PM did not induce changes in the callus WC showing that it was not a limiting factor for the cell growth. Similar results were obtained in embryogenic calli of *Hevea brasiliensis* cultured on medium enriched with sucrose or maltose (Blanc et al. 2002). However, the same authors reported an increment of WC if glucose or fructose was added alone to the medium.

Autoclaving of PM did not determine a sucrose hydrolysis and this result was in agreement with Pan and Staden (1999). After 1 week of culture, an accumulation of both glucose and mostly fructose within PM at the expense of sucrose was observed, as already reported in *Phaseolus vulgaris* (Botha and O'Kennedy 1998) and *Nicotiana tabacum* (Obata-Sasamoto and Thorpe 1982). The callus presence on PM promoted sucrose hydrolysis showing that extracellular *invertases* were involved in the process as

reported in the cell culture of *Beta vulgaris* (Shin et al. 2003). Glucose was preferably imported and metabolized than fructose as reported by Botha and O'Kennedy (1998) and Blanc et al. (2002). The different uptake of hexoses into the callus could depend on the inhibitory effect of glucose on the fructose transporter (Stanzel et al. 1988). However, the results obtained here showed that the total NSC content was in balance in the PM-callus system (a positive correlation was found) indicating that carbon availability was not a limiting factor for callus growth.

The calli had always a lower $\Psi\pi$ than that of the surrounding medium, thus originating an osmotic gradient (i.e. osmotic flow) from PM toward the callus. The osmotic flow might be involved both in the maintenance of an optimal water content (i.e. high turgor pressure) within the cells and in the supply of carbon and nutrient to support growth.

Before starting the experiment, the calli, grown on PM with 30 g l^{-1} of sucrose, had a $\Psi\pi_{callus}$ of -0.75 ± 0.10 MPa, on average. When the trial started (day 0), the calli was placed on different sucrose concentration and the $|\Psi\pi_{callus\text{-}medium}|$ ranged from 0.39 MPa (SF PM) to 0.05 MPa (30 PM). The osmotic adjustments took place rapidly in only 1 week ($|\Psi\pi_{callus\text{-}medium}|$ was 0.2 MPa on average in all PM) showing a fast response to osmotic pressure of PM, independently to initial sucrose concentration. However, at this time a positive RGR was only recorded in the calli cultured on 30 PM demonstrating that high sucrose concentration allowed them to use carbon both to adjust their $\Psi\pi$ and to restore the growth. On the contrary, calli growing on 10 and 20 PM have employed preferably the available carbon only to adjust their $\Psi\pi$ and a similar behaviour was recorded on SF PM. On the basis of these results it is possible to postulate that after the initial osmotic adjustments, a $|\Psi\pi_{callus\text{-}medium}|$ of 0.2 MPa was sufficient to support the osmotic flow from PM to callus enabling its growth. Similarity, in *Nicotiana tabacum* culture a high callus growth was obtained replacing a part of sucrose with mannitol, which was not metabolized but only used for osmotic regulation (Brown et al. 1979).

It was chosen to evaluate the mRNA accumulation of B-type cyclin genes for their fundamental role in the mitosis (Fowler et al. 1998): they were used as molecular marker of cell division. As expected, the mRNA accumulation of B-type cyclin genes in the calli on SF PM showed a trend different from that measured in the calli on the sucrose enriched PM. It has been reported that *CycB1.1* is more transcribed in cultured cells of *Arabidopsis* if the growth medium is supplemented with auxins and cytokinins and less transcribed when sucrose is also added. In summary, sucrose presence has a negative influence on the B-type cyclin genes transcription (Richard et al. 2002). In fact, transcript accumulation of mRNA of *CycB* genes was higher in the calli on 20 PM than on 30 PM. A basal level of sucrose in PM was anyway necessary for allowing cell division. In fact, after 3 weeks of culture the calli on SF PM did not show an accumulation of mRNA transcribed from *CycB* genes and they did not have mitotic activity; on the contrary, these calli had an accumulation of mRNA transcribed from housekeeping genes indicating that they were still alive but entered in a quiescence state. In summary, transcript accumulation level of mRNA of B-type cyclin genes in calli obtained from secondary meristematic tissue of *Populus alba* did not show a direct proportionality to sucrose content in PM: higher sucrose content in PM (and in the calli) did not imply higher mRNA accumulation of *CycB* genes. The growth data compared to the transcript accumulation trend suggested that a threshold effect rather than a quantitative regulation model governed the relation between *CycB* gene transcription and cell division.

The mRNA accumulation of diverse isoforms of B-type cyclin genes was also interesting. *CycB1.3-4* and *CycB2.1* had a higher gene transcription compared to other isoforms. Probably these isoforms are the most active in dedifferentiated tissues and in further trials they can directly be analysed as marker of cell proliferation.

In conclusion, a relation between cell proliferation and osmotic potential of the calli was found. This relation was maintained by the NSC concentration in PM. Carbon availability was fundamental for the growth recovery after subculturing and for cellular osmoregulation and, on the basis of these results, the hypothesis that only sucrose regulated cell division and proliferation in the callus culture was rejected. NSC were essential for callus growth, osmotic potential adjustments and cell division. The absence of sucrose in the PM determined the entry of the calli into a quiescent state, as confirmed by mRNA accumulation pattern of *CycB* and housekeeping genes.

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Author contribution statement AG and GE conceived and designed the research. ST, ADC, GE and MLT performed all the biometric, biochemical, and molecular analyses. ST, GE, AG, ADC and CC analysed data. All Authors drafted and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Asemota O, Eke CR, Odewale JO (2007) Date palm (*Phoenix dactylifera* L.) in vitro morphogenesis in response to growth regulators, sucrose and nitrogen. Afr J Biotechnol 6(20):2353–2357

Berta M, Giovannelli A, Sebastiani F, Camussi A, Racchi ML (2010) Transcriptome changes in the cambial region of poplar (*Populus alba* L.) in response to water deficit. Plant Biol 12(2):341–354. doi:10.1111/j.1438-8677.2009.00320.x

Beritognolo I, Harfouche A, Brilli F, Prosperini F, Gaudet M, Brosché M, Salani F, Kuzminsky E, Auvinen P, Paulin L, Kangasjärvi J, Loreto F, Valentini R, Mugnozza GS, Sabatti M (2011) Comparative study of transcriptional and physiological responses to salinity stress in two contrasting *Populus alba* L. genotypes. Tree Physiol 31(12):1335–1355. doi:10.1093/treephys/tpr083

Blanc G, Lardet L, Martin A, Jacob JL, Carron MP (2002) Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Müll. Arg.). J Exp Bot 53(373):1453–1462. doi:10.1093/jexbot/53.373.1453

Brown DCW, Leung DWM, Thorpe TA (1979) Osmotic requirement for shoot formation in tobacco callus. Physiol Plant 46(1):36–41. doi:10.1111/j.1399-3054.1979.tb03182.x

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 4:14. doi:10.1186/1471-2229-4-14

Botha FC, O'Kennedy MM (1998) Carbohydrate utilization by cell suspension cultures of *Phaseolus vulgaris*. Physiol Plant 102:429–436. doi:10.1034/j.1399-3054.1998.1020311

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Report 11(2):113–116. doi:10.1007/BF02670468

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103(4):551–560. doi:10.1093/aob/mcn125

Confalonieri M, Balestrazzi A, Bisoffi S, Carbonera D (2003) In vitro culture and genetic engineering of *Populus* spp.: synergy for forest tree improvement. Plant Cell Tissue Organ Cult 72(2):109–138. doi:10.1023/A:1022265504775

Confalonieri M, Belenghi B, Balestrazzi A, Negri S, Facciotto G, Schenone G, Delledonne M (2000) Transformation of elite white poplar (*Populus alba* L.) cv. 'Villafranca' and evaluation of herbicide resistance. Plant Cell Rep 19(10):978–982. doi:10.1007/s002990000230

Delledonne M, Allegro G, Belenghi B, Balestrazzi A, Picco F, Levine A, Zelasco S, Calligari P, Confalonieri M (2001) Transformation of white poplar (*Populus alba* L.) with a novel *Arabidopsis thaliana* cysteine proteinase inhibitor gene and analysis of insect pest resistance. Mol Breed 7(1):35–42. doi:10.1023/A:1009605001253

Demeter Z, Kanalasa P, Máthéa C, Csekeb K, Szöllösi E, Hamvasa M, Jámbrika K, Kissa Z, Mészáros I (2014) Osmotic stress responses of individual white oak (*Quercus* section, *Quercus* subgenus) genotypes cultured in vitro. J Plant Physiol 171:16–24. doi:10.1016/j.jplph.2013.09.013

De Veylder L, Joubes J, Inzè D (2003) Plant cell cycle transitions. Curr Opin Plant Biol 6(6):536–543. doi:10.1016/j.pbi.2003.09.001

Dong Q, Zhao Y, Jiang H, He H, Zhu S, Cheng B, Xiang Y (2011) Genome-wide identification and characterization of the cyclin gene family in *Populus trichocarpa*. Plant Cell Tissue Organ Cult 107:55–67. doi:10.1007/s11240-011-9957-z

Eckstein A, Zieba P, Gabrys H (2012) Sugar and light effects on the condition of photosynthetic apparatus of *Arabidopsis thaliana* cultured in vitro. J Plant Growth Regul 31(1):90–101. doi:10.1007/s00344-011-9222-z

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucl Acids Res 32(5):1792–1797. doi:10.1093/nar/gkh340

Fowler MR, Eyre S, Scott NW, Slater A, Elliott MC (1998) The plant cell cycle in context. Mol Biotechnol 10:123–153. doi:10.1093/jxb/eru188

Giovannelli A, Emiliani E, Traversi ML, Deslauriers A, Rossi S (2011) Sampling cambial region and mature xylem for non structural carbohydrates and starch analyses. Dendrochronologia 29:177–182. doi:10.1016/j.dendro.2011.01.001

Harashima H, Dissmeyer N, Schnittger A (2013) Cell cycle control across the eukaryotic kingdom. Trends Cell Biol 23(7):345–356. doi:10.1016/j.tcb.2013.03.002

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8(2):R19. doi:10.1186/gb-2007-8-2-r19

Howard A, Pelc SR (1986) Synthesis of deoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Int J Radiat Biol 49(2):207–218. doi:10.1080/09553008514552501

Kirkan MB, Gardner WR, Gelroff GC (1972) Regulation of cell division and cell enlargement by turgor pressure. Plant Physiol 49(6):961–962

Khayri JM, Bahrani AM (2002) Callus growth and proline accumulation in response to sorbitol and sucrose-induced osmotic stress in rice. Biol Plant 45(4):609–611. doi:10.1023/A:1022380827034

Javed F, Ikram S (2008) Effect of sucrose induced osmotic stress on callus growth and biochemical aspects of two wheat genotypes. Pak J Bot 40(4):1487–1495

Jayaraman S, Daud NH, Halis R, Mohamed R (2014) Effects of plant growth regulators, carbon sources and pH values on callus induction in *Aquilaria malaccensis* leaf explants and characteristics of the resultant calli. J For Res 25(3):535–540. doi:10.1007/s11676-014-0492-8

Iannucci A, Russo M, Arena L, Fonzo N, Martiniello P (2002) Water deficit effects on osmotic adjustment and solute accumulation in leaves of annual clovers. Eur J Agron 16(2):111–122. doi:10.1016/S1161-0301(01)00121-6

Lambardi M, Fabbri A, Caccavale A (2000) Cryopreservation of white poplar (*Populus alba* L.) by vitrification of in vitro-grown shoot tips. Plant Cell Rep 19(3):213–218. doi:10.1007/s002990050001

Luisi A, Giovannelli A, Traversi ML, Anichini M, Sorce C (2014) Hormonal response to water deficit in cambial tissue of *Populus alba* L. J Plant Growth Regul 33(3):489–498. doi:10.1007/s00344-013-9401-1

Marron N, Ricciotti L, Bastien C, Beritognolo I, Gaudet M, Paolucci I, Fabbrini F, Salani F, Dillen SY, Ceulemans R, Pinel MPC, Taylor G, Scarascia-Mugnozza G, Sabattia M (2010) Plasticity of growth and biomass production of an intraspecific *Populus alba* family grown at three sites across Europe during three growing seasons. Can J Res 40:1887–1903. doi:10.1139/X10-113

Matsumoto T, Okunishi K, Nishida K, Noguchi M, Tamaki E (1971) Studies on the culture conditions of higher plant cells in suspension culture. Part II. Effect of nutritional factors on the growth. Agric Biol Chem 35(4):543–551. doi:10.1271/bbb1961.35.543

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15(3):473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Mustafa NR, De Winter W, Van Iren F, Veerporter R (2011) Initiation, growth and cryopreservation of plant cell suspension cultures. Nat Protoc 6(6):715–742. doi:10.1038/nprot.2010.144

Nieuwland J, Scofield S, Murray JAH (2009) Control of division and differentiation of plant stem cells and their derivatives. Elsevier Semin Cell Dev Biol 20:1134–1142. doi:10.1016/j.semcdb.2009.09.011

Nuutila AM, Kurtén U, Kauppinen V (1991) Optimization of sucrose and inorganic nitrogen concentrations for somatic embryogenesis of birch (*Betula pendula* Roth.) callus cultures: a statistical approach. Plant Cell Tissue Organ Cult 24(2):73–77. doi:10.1007/BF00039733

Obata-Sasamoto H, Thorpe TA (1982) Cell wall invertase activity in cultured tobacco tissues. Plant Cell Tissue Organ Cult 2:3–9. doi:10.1007/BF00033546

Pallara G, Giovannelli A, Traversi ML, Camussi A, Racchi ML (2012) Effect of water deficit on expression of stress-related genes in the cambial region of two contrasting poplar clones. J Plant Growth Regul 31:102–112. doi:10.1007/s00344-011-9223

Pan MJ, Staden J (1999) Effect of activated charcoal, autoclaving and culture media on sucrose hydrolysis. Plant Growth Regul 29:135–141. doi:10.1023/A:1006238300890

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45. doi:10.1093/nar/29.9.e45

Richard C, Lescot M, Inzè D, De Veylder L (2002) Effect of auxin, cytokinin and sucrose on cell cycle gene expression in *Arabidopsis thaliana* cell suspension cultures. Plant Cell Tissue Organ Cult 69:167–176. doi:10.1023/A:1015241709145

Riou-Khamlichi C, Menges M, Healy JMS, Murray JAH (2000) Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol Cell Biol 20(3):4513–4521

Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. Plant Cell 14(1):185–205. doi:10.1105/tpc.010455

Shin KS, Chakrabarty D, Ko JY, Han SS, Paek KY (2003) Sucrose utilization and mineral nutrient uptake during hairy root growth of red beet (*Beta vulgaris* L.) in liquid culture. Plant Growth Regul 39:187–193. doi:10.1023/A:1022525308389

Stanzel M, Sjolund RD, Komor E (1988) Transport of glucose, fructose and sucrose by *Streptanthus tortuosus* suspension cells. I. Uptake at low sugar concentration. Planta 174:201–209. doi:10.1007/BF00394773

Stals H, Inzè D (2001) When plant cells decide to divide. Trends Plant Sci 6(8):359–364. doi:10.1016/S1360-1385(01)02016-7

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Tisserat B (1982) Factors involved in the production of plantlets from date palm callus cultures. Euphytica 31:201–214. doi:10.1007/BF00028323

Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr Opin Biol 9(2):189–195. doi:10.1016/j.pbi.2006.01.019