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BRIEF COMMUNICATION

Wood ontogeny during *ex vitro* acclimatization in micropropagated hybrid poplar clones

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Abstract

Wood ontogeny patterns were determined during the *ex vitro* acclimatization period in micropropagated plantlets of hybrid poplar clones T-14 [*Populus tremula* × (*Populus* × *canescens*)] and T-50 [(*Populus* × *canescens*) × *Populus tremula*]. The temporal course of developmental changes in the woody tissue was characterized on a weekly basis starting from the day of transfer to the *ex vitro* environment until full acclimatization was achieved on day 28. *In vitro* rooted plantlets had already initiated lignification of secondary xylem cells. The greatest increase in the amount of woody tissue was observed on days 21 and 28. At the end of the acclimatization period, T-14 plantlets contained on average 41.4 % of secondary xylem tissue compared to 30.3 % found in T-50 plantlets. During the course of acclimatization, both clones displayed identical patterns of vessel lumen size distribution from small vessel lumen area to large vessel lumen area. This pattern differs from the characteristic diffuse-porous pattern of approximately even-sized vessel lumen area distribution typical of mature wood. At the end of acclimatization, the differences in vessel lumen area and relative conductivity between the clones were negligible. Development of secondary xylem tissue during *ex vitro* acclimatization promotes the establishment of vigorous regenerants with stems that show increased bending strength and stiffness.

Additional key words: *Populus* × *canescens*, *Populus tremula*, relative hydraulic conductivity, secondary xylem, vessel lumen area.

Grey poplar [*Populus* × *canescens* (Ait.) Sm.] is a dioecious tree widespread in central Europe that originated from the spontaneous hybridization between the white poplar (*P. alba*) and the aspen (*P. tremula*). It grows best in damp ground, near rivers, and water meadows. In addition, the grey poplar is also tolerant toward oscillations in underground water levels and capable of growth in acidic soils, heavy clay soils, or even in fens. These attributes make this tree a very promising candidate for commercial planting within the constraints of global warming and also for utilization as the parental species in artificial hybridization with other poplar clones for the production of new genotypes with an altered content of cell wall constituents for the pulp and paper industry.

Micropropagation of forest trees is a powerful tool for the production of true-to-type clonal plants for afforestation and plantation programs. The acclimatization phase is often a critical period when plantlets have to correct both anatomical and physiological disorders originating *in vitro*. To improve the rate of survival and the physiological functioning of micropropagated plantlets, procedures such as a gradual decrease of relative humidity, the removal of sugars and an increase in CO_2 concentration can be applied during *ex vitro* acclimatization (reviewed in Pospíšilová *et al*. 1999). Most investigations have studied the changes in leaf anatomy, water balance, antioxidant metabolism, leaf gas exchange, and chlorophyll fluorescence after transfer to the *ex vitro* environment. These studies were carried out in herbaceous species (Van Huylenbroeck *et al*. 1998, Jeon *et al*. 2006, Aragón *et al*. 2010), as well as in woody

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plants (Carvalho *et al*. 2001, Purohit *et al*. 2002, Park *et al*. 2011). On the other hand, our knowledge of both the initiation of wood formation under *in vitro* conditions and the ontogeny of woody tissue during the transition of micropropagated timber species from *in vitro* culture to the acclimatization phase is still fragmentary. Unlike *in vitro* formed leaves that usually fall during or shortly after *ex vitro* acclimatization to be replaced by leaves formed *ex vitro*, stem functioning runs parallel throughout *in vitro* and *ex vitro* conditions. The aim of this study was to investigate developmental changes during the *ex vitro* acclimatization phase in relation to the secondary xylem formation, vessel lumen sizes, vessel densities, and relative conductivities in two artificially bred hybrids between grey poplar and aspen. The time-course of these changes was assessed using clonal plant material grown at decreasing relative humidity (RH), constant photosynthetic photon flux density, and constant temperature to avoid imbalances in cambial cell division and to reduce the environmental effects on the rate of lignification.

Two mature, more than 30-year-old hybrid poplar trees T-14 [*Populus tremula* 70 × (*Populus* × *canescens* 23)] and T-50 [*Populus* × *canescens* 26) × *Populus tremula* 32] clones were selected for the study. The T-14 clone displays a significantly higher content of cellulose than that of the T-50 clone (47.33 ± 0.19 % *versus* 45.41 ± 0.22 %). On the other hand, the T-50 clone contains a lower amount of Klason lignin than that of the T-14 clone (16.69 ± 0.26 % *versus* 17.37 ± 0.13 %). Sprouting axillary buds were used for the *in vitro* culture establishment according to the procedure described in Ďurkovič and Lux (2010). Disinfected explants were placed onto woody plant medium (WPM, Lloyd and McCown 1980) solidified with agar (6 g dm^{-3}, *Sigma*, St. Louis, MO, USA). Sucrose (20 g dm^{-3}) was added and pH was adjusted to 5.6 - 5.8 with 1 M KOH. Shoot cultures were multiplied on WPM supplemented with 1.33 μM 6-benzylaminopurine and 0.27 μM 1-naphthaleneacetic acid (NAA). Cultures were regularly sub-cultured every 4 weeks. For adventitious rooting, microshoots more than 1.5 cm long were excised from proliferating shoot cultures and transferred to half-strength WPM supplemented with 0.53 μM NAA for 4 weeks. Cultures were maintained at day/night temperatures of 24/20 ± 1 °C, a 16-h photoperiod, irradiance of 50 μmol m^{-2} s^{-1} (cool white fluorescent tubes), and relative humidity of 99 ± 1 % inside test tubes.

On day 0, the culture medium was carefully washed off the roots of *in vitro* rooted plantlets and the plantlets were transferred to pots filled with a sterile mixture of soil and peat. Potted plantlets were transferred to a controlled environment room and covered with transparent glass flasks. Starting on day 20 after transfer, the glass cover was opened stepwise for 7 d to decrease the relative humidity. On day 27, the glass cover was removed completely. Except for relative humidity, plantlets were acclimatized to the *ex vitro* environment under conditions similar to those used during *in vitro* culturing, *i.e*. day/night temperatures of 24/20 ± 1 °C, 16-h photoperiod, and irradiance of 50 μmol m^{-2} s^{-1}. Fully acclimatized plantlets were then transferred to a greenhouse. Experiments were made on stem cross-sections sampled at five different stages of *ex vitro* acclimatization: day 0 corresponding to *in vitro* rooted plantlets at 99 ± 1 % RH, 7 d after transfer at 99 ± 1 % RH, 14 d after transfer at 99 ± 1 % RH, 21 d after transfer at 81 ± 2 % RH, and 28 d after transfer at 50 ± 2 % RH. RH was measured using a *Commeter D3120* thermo-hygrometer (*Comet System*, Rožnov pod Radhoštěm, Czech Republic). The acclimatization procedure, described previously in Kang *et al*. (1992), was optimalized by the extension of the 3-week acclimatization period to 4 weeks, and survival rate was 96 %.

At each stage, stem segments were sampled 0.5 cm upwards from the root-stem junction from four different rooted shoots per clone. Segments were fixed overnight in 3.5 % glutaraldehyde in a 0.1 M sodium phosphate buffer, pH 7.2, at 4 °C. Afterwards, segments were dehydrated, cleared with xylene, embedded in paraffin wax, sectioned, deparaffinized, and rehydrated as described in Ďurkovič and Mišalová (2009). For safranin-alcian blue double staining, sections were first stained with 1 % safranin in 50 % ethanol for 1 min, followed by 1 % alcian blue in 0.05 % acetic acid containing 0.04 % formaldehyde for 1 min (Marjamaa *et al*. 2003), rinsed with water, dehydrated in an ascending ethanol series, rinsed with xylene, mounted in a drop of Clarion mounting medium beneath a coverslip, and observed with an *Olympus BX50F* microscope (*Olympus Europa*, Hamburg, Germany). Quantitative data of wood parameters (areas of pith cells, secondary xylem, bark, and vessel lumens) were determined with image analysis software (*NIS-Elements AR 3.0*, *Laboratory Imaging*, Prague, Czech Republic). Vessel area percentage was calculated as the ratio of the area occupied by vessels to the area of the secondary xylem multiplied by 100. Relative conductivity was calculated according to Zimmermann (1983) as the fourth power of the equivalent circle diameter of vessel lumen (the equivalent circle diameter is the diameter of the circle having the same area as the measured cell). Measurements were made on four different cross-sections per clone at each stage as described in Ďurkovič and Mišalová (2009). Data from all variables were initially tested for normality. The distributions of vessel lumen areas and relative conductivities were skewed to the left, therefore a logarithmic transformation was used before statistical analysis. Data were then subjected to two-way analysis of variance and Duncan's multiple range test was used for separation of means.

Wood formation is a complex and dynamic process that consists of the successive addition of secondary xylem which differentiates from the vascular cambium. Temporal patterns of secondary xylem formation and growth in both T-14 and T-50 hybrid poplar clones

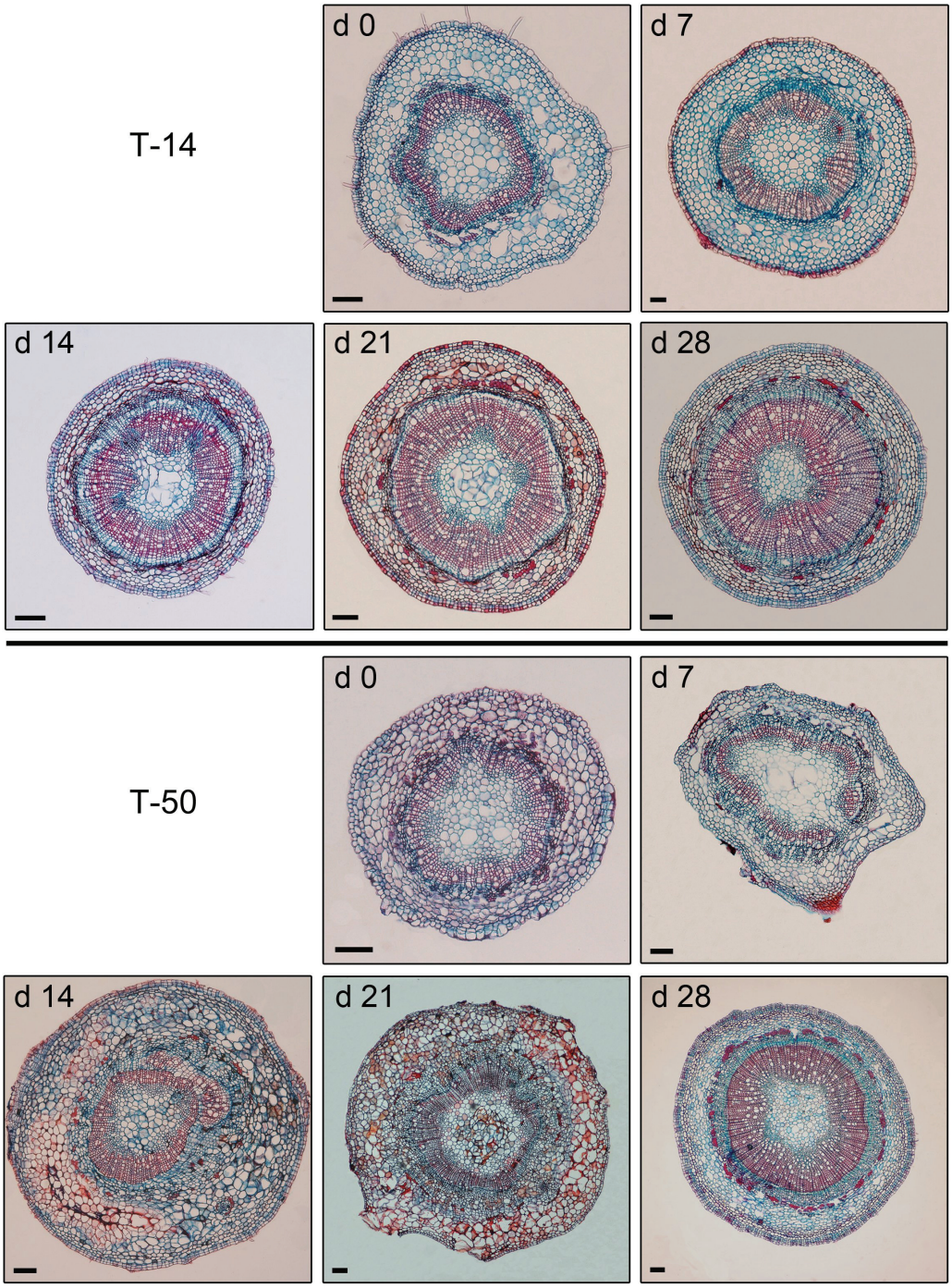

Fig. 1. Secondary xylem growth patterns during *ex vitro* acclimatization of hybrid poplar clones T-14 and T-50, respectively. *Scale bars* = 100 μm.

during *ex vitro* acclimatization are presented in Fig. 1. Double staining of stem cross-sections revealed that wood formation was initiated before the transfer from *in vitro* conditions to the *ex vitro* environment. On day 0, several layers of lignified wood elements were already produced by cells of a complete cambial ring in both clones. Lignin deposition was observed even in secondary phloem fibers. In this stage, higher proportions of secondary xylem tissue were found in T-14 plantlets (12.2 % for T-14 *versus* 8.1 % for T-50, Table 1). The amount of woody tissue gradually increased during all stages of *ex vitro* acclimatization. Significant differences between poplar clones were observed on days 0, 14, and 28 after transfer. Fully acclimatized T-14 plantlets contained on average 41.4 % of secondary xylem tissue compared to 30.3 % found in T-50 plantlets. Previous

studies indicated that new roots, formed during the rooting inductive phase and growing in the presence of exogenous auxin, are the source of signals for lignification, and presumably for wood formation, in stems (Bisbis *et al*. 2003, Hatzilazarou *et al*. 2006). This may explain why wood formation in hybrid poplars was initiated during the *in vitro* rooting phase. But, in the middle period of the rooting phase, the cambial ring in the stem was not yet completed and the clusters of secondary xylem cells were associated exclusively with the fascicular cambium. On day 0 of *ex vitro* transfer, proportions of secondary xylem tissue occupied within the stem area, have been reported to be lower (6.3 % in *Sorbus domestica*) or higher (15.0 % in *Morus nigra*) than the above mentioned rates found in hybrid poplar clones (Ďurkovič and Mišalová 2009, Mišalová *et al*. 2009). In the case of slow growing *Sorbus domestica* with a diffuse-porous wood, lignified cells of secondary xylem were only associated with the fascicular cambium (Ďurkovič and Mišalová 2009). Experiments with hybrid poplar clones showed that secondary xylem was produced by a complete cambial ring and arranged in circular layers on day 0. Thus, following transfer to the *ex vitro* environment, the fast height growth of hybrid poplars may be supported adequately by the earlier secondary growth. At the end of the acclimatization period, both hybrid poplar clones produced higher amounts of woody tissue within the stem area than those of the *ex vitro* acclimatized *Sorbus domestica* and *Morus nigra* plantlets (41.4 % for T-14 and 30.3 % for T-50 *versus* 14.8 % for *Sorbus domestica* and 27.5 % for *Morus nigra* with a ring-porous wood, respectively) even though their acclimatization took twice as long as that of the hybrid poplar taxa (Ďurkovič and Mišalová 2009, Mišalová *et al*. 2009).

In both poplar clones, cell wood composition varied during the acclimatization period. Secondary xylem of *in vitro* rooted plantlets consisted mainly of vessels, parenchyma, and fibers. The differentiated uniseriate parenchyma ray was clearly observed on day 14. Furthermore, the thick cell walls of several fibers suggested the occurrence of libriform ones. With regard to the log-transformed vessel lumen areas, both clones had comparable mean dimensions at the end of the *in vitro* period and at the end of acclimatization, but the dynamics of their changes during acclimatization were different (Table 1). *In vitro* rooted plantlets were characterized by small vessel lumen areas with the maximum values of 194 μm^2 for T-14 and 199 μm^2 for T-50, respectively. The T-14 largest vessel lumen area (681 μm^2) occurred on day 21 while in the T-50 clone, the largest vessel lumen area (652 μm^2) was observed 7 d later at the end of acclimatization. An identical pattern of vessel lumen area distribution, from a small to a large one during *ex vitro* acclimatization, was also described for micropropagated *Sorbus domestica* (Ďurkovič and Mišalová 2009) and for micropropagated *Morus nigra* (Mišalová *et al*. 2009) plantlets. But when comparing the maximum vessel lumen area dimensions in diffuse-porous woods of hybrid poplar taxa and *Sorbus domestica* under acclimatization conditions, the differences were obvious. The largest vessel lumen area in acclimatized *Sorbus domestica* plantlets was not larger than 242 μm^2 (Ďurkovič and Mišalová 2009).

Table 1. Changes in the examined wood traits (see text for details) during *ex vitro* acclimatization of hybrid poplar clones. Data represent means ± SD. Mean values followed by the same letters within the same row are not significantly different at the 0.05 level of significance (Duncan's multiple range test).

Parameter	[d]	T-14	T-50
Proportion of secondary xylem [%]	0	12.15 ± 1.55 a	8.10 ± 2.36 b
	7	15.19 ± 4.57 a	11.57 ± 3.36 a
	14	23.21 ± 8.45 a	12.47 ± 2.03 b
	21	36.49 ± 14.62 a	20.31 ± 10.36 a
	28	41.38 ± 6.20 a	30.25 ± 5.52 b
Vessel lumen area [log]	0	1.87 ± 0.13 a	1.87 ± 0.14 a
	7	1.91 ± 0.17 a	1.91 ± 0.19 a
	14	1.95 ± 0.18 b	2.00 ± 0.21 a
	21	2.12 ± 0.26 a	2.05 ± 0.28 b
	28	2.11 ± 0.27 a	2.11 ± 0.26 a
Vessel area [%]	0	5.14 ± 2.13 a	6.97 ± 2.07 a
	7	8.70 ± 1.37 a	7.83 ± 1.48 a
	14	9.59 ± 2.28 a	10.79 ± 1.85 a
	21	10.51 ± 2.45 a	12.76 ± 3.92 a
	28	8.75 ± 1.69 b	12.43 ± 1.98 a
Relative conductivity [log]	0	3.95 ± 0.26 a	3.96 ± 0.27 a
	7	4.03 ± 0.33 a	4.03 ± 0.38 a
	14	4.10 ± 0.37 b	4.20 ± 0.41 a
	21	4.46 ± 0.51 a	4.31 ± 0.55 b
	28	4.44 ± 0.54 a	4.44 ± 0.51 a

Vessel area percentage increased from day 0 when minimal values were found (5.1 % for T-14 and 7.0 % for T-50, respectively) up to day 21 when maximum values were found (10.5 % for T-14 and 12.8 % for T-50, respectively). On day 28, this parameter either decreased slightly (T-14) or remained steady (T-50) indicating an increased growth and differentiation of other cell wood elements, mainly fibers and parenchyma ray cells. Significant differences between clones were distinguishable at the end of acclimatization (Table 1). Data available for the vessel area percentage in acclimatized *Sorbus domestica* and *Morus nigra* plantlets showed a marked contrast to poplar hybrids. In *Sorbus domestica*, the values of this parameter increased and reached the maximum value at the end of the acclimatization period (3.1 %), whereas the maximum vessel area percentage in *Morus nigra* plantlets was found on day 7 after transfer (19 %) and the values remained steady (~16 %) until the end of acclimatization (Ďurkovič and Mišalová 2009, Mišalová *et al*. 2009). Different results and behaviour of this parameter reflect differences among the various taxa in the growth rates and the nature of wood.

With regard to the log-transformed theoretical relative

conductivities, dynamics of developmental changes in this trait was similar to that of vessel lumen sizes with non-significant differences between clones at the end of acclimatization (Table 1).

Anatomical features of the differentiating secondary xylem, which were examined in this study, are of great interest. Changes in the anatomical characteristics of vessels can alter water hydraulic conductance in the stem. In vessel-bearing angiosperms, the influence of vessels on wood density can be decomposed into two principal components: vessel area (the transverse lumen area of individual vessels) and vessel density (the vessel area percentage). Vessel lumen area strongly affects the capacity of wood to conduct water (Zimmermann 1983) whereas the significance of vessel density lies in its relationship to the interconnectedness of the vascular system within a stem (Preston *et al*. 2006). Our study revealed a gradual increase in the growth rates of secondary xylem formation, vessel lumen areas, and relative conductivities for both clones during acclimatization. At the end of the acclimatization phase, the differences in vessel lumen area and relative conductivity between the examined clones, differing in the lignin and cellulose content, were negligible. Thus, a more efficient water transport system, which plays a crucial role in rapidly growing plants such as poplars, was established in both clones compared to that found at the end of the *in vitro* culture period. Development of secondary xylem during *ex vitro* acclimatization promoted the establishment of vigorous regenerants of these valuable hybrid poplar clones, primarily selected for their possible utilization in the pulp and paper industry.

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