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Peroxidase, polyphenol oxidase activity and total phenolic concentration in birch (*Betula pendula*) *in vitro* shoots during rejuvenation

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Abstract

Tissue recalcitrance remains a major problem limiting woody plant propagation using *in vitro* biotechnology methods. Increased generation of reactive oxygen species and high phenol concentration in woody tissues are considered as the main reasons for tissue recalcitrance in tissue culture. In this study, oxidative enzyme activity and total phenolic concentration were analyzed *in vitro* in both rejuvenated and mature shoots of birch. The effect of two factors – subculture time and medium sucrose concentration – on peroxidase and polyphenol oxidase activity, and total phenolic concentration in rejuvenated shoots were determined as possible rejuvenation-affecting factors. The results showed that mature shoots had increased peroxidase and polyphenol oxidase activity in shoot apices and stems but higher phenolic concentration in leaves as compared to rejuvenated shoots. Prolonged subculture time had significant effect on peroxidase activity and polyphenol oxidase activity in shoot apices, and thus this factor did not affect maturation. However, high sucrose concentration significantly increased total phenolic concentration in leaves and polyphenol oxidase activity in all plant parts, thus enhancing shoot maturation and recalcitrance *in vitro*.

Key words: *Betula pendula*, phenolics, peroxidase, polyphenol oxidase, rejuvenation, sucrose.

Introduction

Micropropagation is an effective vegetative technique to obtain clonal material of desired woody plant varieties or superior genotypes. Tissue recalcitrance is a major factor limiting mature woody plant micropropagation and is described as inability of tissues to respond to *in vitro* stimulus to reach morphogenic competence and totipotent capacity (Benson 2000). Recalcitrance is attained gradually as a tree ages and shifts to its mature phase, and is encompassed with other significant physiological changes, like seasonal shoot growth pattern, reduced growth and rooting capacity as well as production of secondary metabolites (McCown 2000). The level of recalcitrance for a particular explant is influenced by both endogenous and exogenous factors (Bonga et al. 2010).

One of the main physiological factors affecting tissue culture recalcitrance is oxidative stress (Benson 2000). Mature woody plants usually have elevated oxidative enzyme activity and contain higher amounts of phenolic compounds as compared to juvenile plants of the same species (Warrier et al. 2013). When such tissues are introduced in tissue culture, wounding and other stresses during cultivation (osmotic shock, nitrogen toxicity, altered gas composition, hormonal imbalance) cause rapid production of reactive oxygen species, as a result leading to disorganization of cell structures and oxidation of phenolic compounds, appearing as tissue browning, necrosis and death (Benson 2000).

Elevated oxidative enzyme activity and higher phenolic concentration has been shown to be correlated with low tissue responsiveness in tissue culture (Laukkanen et al. 1999). Despite recent advances in elucidation of the physiological and molecular basis of woody plant development and *in vitro* regeneration, an exact mechanism of tissue recalcitrance *in vitro* and ways to overcome it remains poorly understood (Díaz-Sala 2016). Therefore, the aim of this study was to determine oxidative enzyme activity and total phenolic concentration in rejuvenated and mature recalcitrant birch shoots in tissue culture, and to determine the effect of two *in vitro* factors – subculture time and sucrose concentration – on peroxidase, polyphenol oxidase activity and total phenolic concentration in rejuvenated shoots.

Materials and methods

Plant material

Buds from 10 different clones of mature approximately 20-year-old birch (*Betula pendula* Roth) elite trees grown in a progeny trial in the region of Ķegums in Latvia were selected and initiated *in vitro* to obtain shoot cultures. After six months three clones showing both rejuvenated shoots (characterized with high proliferation rate and high rooting

capacity) and mature shoots (characterized with low proliferation rate and zero rooting capacity) were selected for further experiments. Three experimental sets were used, with approximately 30 rejuvenated and 30 mature shoots per each experiment.

(1) Determination of oxidative enzyme activity and total phenolic concentration in rejuvenated and mature birch in vitro shoots. Shoots from each type were cultured on Woody Plant Medium (Lloyd, McCown 1980) supplemented with 0.2 mg L^{-1} zeatin, 20 g L^{-1} sucrose and 6 g L^{-1} agar. pH of the medium was adjusted to 5.8 before autoclaving for 15 min (110 kPa, 121 °C). Cultures were kept at 25 ± 3 °C, with a 16/8 h light/dark period, illumination provided by cool-white fluorescent lamps (photosynthetically active radiation with a photon flux density 140 to 160 µmol m^{-2} s^{-1}). After four weeks shoots were collected for enzyme and total phenolic concentration assay.

(2) The effect of subculture time on oxidative enzyme activity. Rejuvenated shoots were cultured as previously described. During cultivation, shoots were collected for enzyme assay after three subculture time intervals: one week, eight weeks and 11 weeks.

(3) The effect of elevated sucrose concentration on oxidative enzyme activity and total phenol content. Rejuvenated shoots were kept on sucrose concentration 20 and 50 mg L^{-1} for two weeks, part of shoots were used for enzyme extraction and the remaining were cultured for two more weeks with subsequent total phenolic extraction.

Guaiacol peroxidase and polyphenol oxidase assay

Enzyme assay was performed by a modified protocol from Andersone and Ievinsh (2002). Shoots were divided into leaves, shoot apices and stems. Samples (250 mg) from each plant part were ground together with 2.5 mL 25 mmol L^{-1} HEPES/KOH buffer (pH 7.2), containing 1 mmol L^{-1} EDTA and 1% (w/v) polyvinylpolypyrrolidone. Extract was kept 15 min at 4 °C, followed by centrifugation at 13 000 rpm for 20 min. Guaiacol peroxidase and polyphenoloxidase activity was measured spectrophotometrically using a Lambda 25 UV/Vis Spectrometer (PerkinElmer). For peroxidase, the reaction mixture contained 2 mL 50 mmol L^{-1} sodium phosphate (NaH_2PO_4/Na_2HPO_4) buffer (pH 7.0) with 10 mmol L^{-1} guaiacol, 0.5 mL 3 mmol L^{-1} H_2O_2 and 0.02 mL enzyme extract. Activity was measured at 470 nm. For polyphenol oxidase, the reaction mixture contained 3 mL 20 mmol L^{-1} sodium phosphate buffer (pH 7.0) with 25 mmol L^{-1} pyrocathecol and 0.1 mL enzyme extract. Activity was measured at 410 nm. Three biological and three chemical replicates were performed for each treatment. Mean and standard deviation were calculated.

Total phenol concentration assay

Total phenolic concentration assay was performed according to modified protocol from Harald et al. (2012). Leaves were dried at room temperature and grated in fine powder. Samples (250 mg) of plant material were mixed with 50 µL 50% ethanol and centrifuged at 5 000 rpm for 10 min. The supernatant was diluted to 6% using 50% ethanol. Gallic acid was used as a standard at 0.025 to 0.2 mg mL^{-1} to produce a calibration curve (R^2 = 0.996). In each cell of a 96-well microplate, 75 µL H_2O, 25 µL 6% extract or standard and 25 µL 50% Folin–Ciocalteu reagent were added. Solutions were mixed and left for 6 min. Then 100 µL 7% Na_2CO_3 was added to each well, mixed again and left in the dark for 90 min. The absorbance at 620 nm was measured with a Biochrom Asys Expert Plus spectrophotometric microplate reader (Biochrom). Each standard and sample solution was analysed in triplicate, and assayed against a sample control (50% ethanol). Total phenolic concentration was expressed as gallic acid equivalent (GAE; mg g^{-1}).

Results

Significant differences in peroxidase and polyphenol oxidase activity were observed between different plant parts from rejuvenated and mature shoots (Fig. 1). The lowest enzyme activity for both rejuvenated and mature shoots was observed in leaves followed by shoot apices and stems, except for peroxidase activity in shoot apices in mature shoots, which was higher than in stems. Both peroxidase and polyphenol oxidase activity was about 1.6 times lower in mature shoot leaves as compared to rejuvenated shoot leaves, whereas in shoot apices and stems the activity was higher in mature shoots by about 1.3 times for peroxidase and 1.8 times for polyphenol oxidase. In mature shoots, differences in polyphenol oxidase activity were more

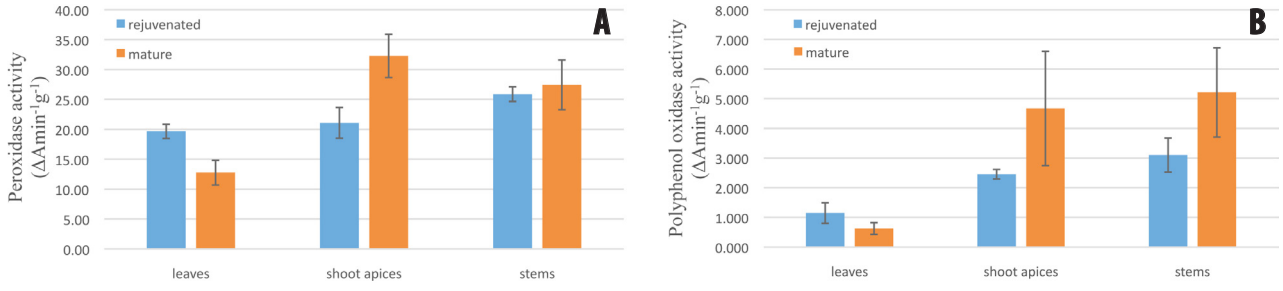

Fig. 1. Peroxidase (A) and polyphenol oxidase (B) activity in different shoot structures of mature and rejuvenated *Betula pendula* shoots. Mean and standard deviation is shown.

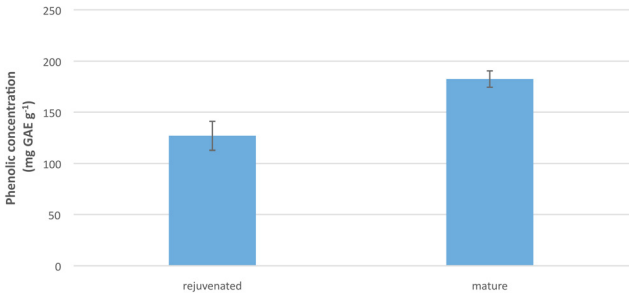

Fig. 2. Total phenolic concentration in leaves of mature and rejuvenated *Betula pendula* shoots. Mean and standard deviation is shown.

pronounced in comparison to peroxidase activity. Results for total phenolic analysis showed that mature shoots had elevated levels of phenolic compounds, having 1.4 times higher phenolic concentration in leaves as compared to leaves of rejuvenated shoots (Fig. 2).

Evaluating the effect of subculture time on enzyme activity, it was observed that peroxidase activity was relatively stable in time in all shoot structures during cultivation, except peroxidase activity in shoot apices, which was about three times higher after 11 weeks as compared to activity after one or eight weeks of subculturing (Fig. 3). In contrast, polyphenol oxidase activity was significantly higher in leaves and stems after one week of subculturing, but was lower and remained relatively stable after eight and 11 weeks.

After two weeks of cultivation in the presence of elevated sucrose concentration in the medium (50 g L^{-1}), contrasting effect on peroxidase and polyphenol oxidase activity was observed (Fig. 4). Peroxidase activity decreased in all structures in the presence of elevated sucrose, with the greatest reduction in leaves and shoot apices, where the activity was about 1.4 times lower than at optimal sucrose concentration. The opposite effect was observed for polyphenol oxidase, where the activity increased in all structures by about 1.3 times. There was also a significant increase in total phenolic concentration in shoots grown on elevated sucrose, which was 2.5 times higher than at optimal sucrose concentration (Fig. 5).

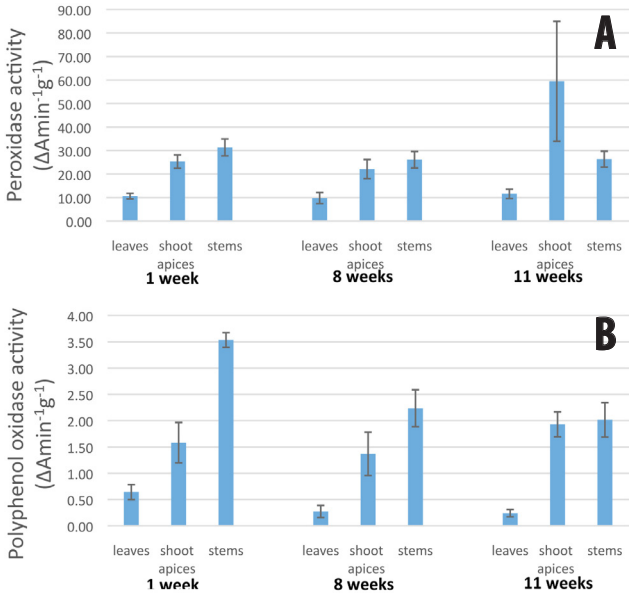

Fig. 3. Peroxidase (A) and polyphenol oxidase (B) activity in different shoot structures in rejuvenated *Betula pendula* shoots at different subculture times. Mean and standard deviation is shown.

Discussion

A variety of different potential explanations for recalcitrance have been published, suggesting that several mechanisms may operate independently, including nutritional and hormonal state, oxidative enzyme activity and genetic and epigenetic factors (Díaz-Sala 2016). In this study we observed elevated peroxidase and polyphenol oxidase activity in mature recalcitrant birch shoots apices and stems as compared to rejuvenated shoots. Oxidative enzyme activity has been shown to be elevated in mature plants and it usually correlated with low tissue responsiveness in tissue culture (Warrier et al. 2013). For example, Andersone and Ievinsh (2002) observed no tissue morphogenesis of *Pinus sylvestris* buds, when peroxidase and polyphenol oxidase levels were higher, compared to increased morphogenesis, when oxidative enzyme activity was lower and remained stable.

High peroxidase activity in mature plants can be

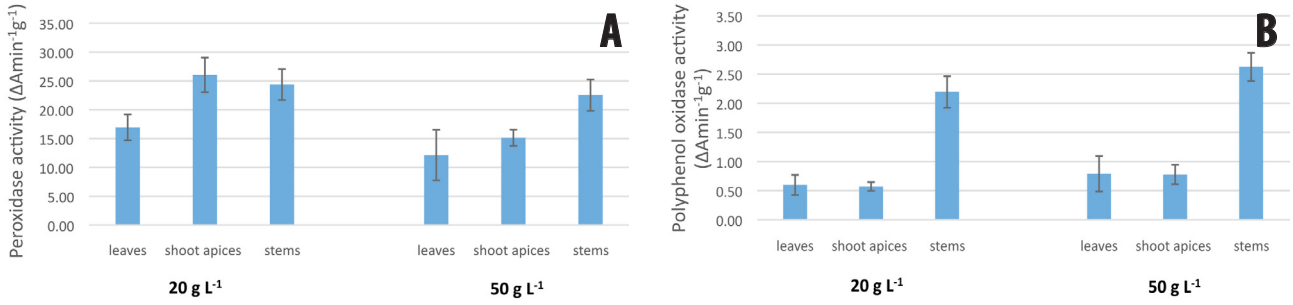

Fig. 4. Peroxidase (A) and polyphenol oxidase (B) activity in different shoot structures in rejuvenated *Betula pendula* shoots cultured on different sucrose concentration. Mean and standard deviation is shown.

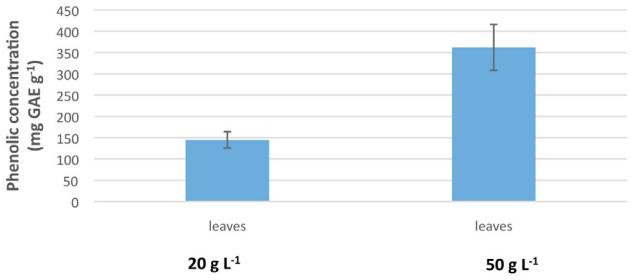

Fig. 5. Total phenolic concentration in leaves of rejuvenated *Betula pendula* shoots cultured on different sucrose concentration. Mean and standard deviation is shown.

attributed to its role in reduction of H_2O_2 and lignification processes (Hiraga et al. 2001). This indicates that the higher peroxidase activity in stems and shoot apices observed in this experiment as compared to leaves could also be attributed to increased lignification in these parts. Lower oxidative enzyme activity in mature shoot leaves could be reason of ageing of leaves and tissue deterioration. H_2O_2 is the most abundant reactive oxygen species in plants and its concentration increases dramatically during the adult phase, as does the concentrations of its scavengers (Du et al. 2015). It is known that redox homeostasis, which is maintained by the precise generation and scavenging of H_2O_2 and other types of reactive oxygen species, interacts with the phytohormone signalling network, thus controlling many aspects of plant growth, differentiation, development and environmental responses (Considine, Foyer 2014). H_2O_2 is a known inducer of gene expression, and recent studies indicate its role in the molecular mechanism of phase change through modulating the expression of phase change master regulator microRNA miR156/157 (Wu et al. 2009). When H_2O_2 or glutathione concentration of suspension cells was altered by specific treatment, the levels of gene *MdMIR156a5* and *MdMIR156a12* transcripts as well as the mature miR156 expression level changed accordingly (Jia et al. 2017).

Polyphenol oxidase activity is also usually elevated in mature tissues and is often observed together with elevated phenolic concentration (Warrier et al. 2013). Plants synthesize phenolics constitutively or as a response to stress. Wounding and other stresses during *in vitro* initiation cause disorganization of tissue or cell structures, resulting in mixing of phenolics, enzymes, and their co-substrates O_2 or H_2O_2, leading to oxidation of phenolic compounds, browning and necrosis (Benson 2002). Both peroxidase and polyphenol oxidase are involved in this process, and thus their activity is usually elevated in tissues rich in phenolics (Vondráková et al. 2016).

In this study polyphenol oxidase activity was higher in mature tissues and showed similar pattern to peroxidase, only the differences between juvenile and mature parts were more pronounced than for peroxidase, making it a better marker for shoot mature state determination. We also observed elevated polyphenol oxidase activity in mature shoots together with higher phenolic concentration, which perhaps caused their recalcitrance. Woody plants, especially *Quercus*, *Pinus* and *Betula*, are rich in tannins that often leads to tissue culture browning and are considered as a major factor for the recalcitrance *in vitro* (Vondráková et al. 2016).

During *in vitro* cultivation, plant tissues are subjected not only to higher oxidative metabolism already present in mature tissues, but to all kinds of other stresses during cultivation, like osmotic shock (resulting from high amount of sucrose), nitrogen toxicity, hormonal imbalance and gas toxicities (high concentration ethylene) etc., that can lead to oxidative stress (Desjardins et al. 2009). For example, long subculture intervals can enhance phenolic synthesis and elevate peroxidase activity (Abohatem et al. 2011). In this study we observed increased peroxidase activity in rejuvenated shoot apices after 11 weeks of cultivation, whereas polyphenol oxidase activity showed only a little increase. Instead, polyphenol oxidase increased after one week of cultivation, indicating wound-induced phenolic synthesis. Therefore, the increased peroxidase activity in shoot apices after 11 weeks could be due to mineral deficiency (caused by prolonged time without transfer to new medium) rather than to maturation. Therefore, the prolonged culture time is not likely to cause maturation and recalcitrance in birch shoots.

Sucrose is known to induce major stress responses *in vitro*, including interaction with hormone signalling pathways, binding to hexokinase that activates a number of stress adaptation responses, inhibition of the photosynthetic electron transport chain and osmotic stress (Desjardins et al. 2009). Sucrose is also known as one of the main factors inducing maturation in plants. The glucose signalling protein in *Arabidopsis thaliana* showed close relationship with the expression of two genes *MIR1256A* and *MIR156C* (Yu et al. 2013). In this study elevated sucrose concentration caused decrease in peroxidase activity in all plant parts. Various abiotic stress factors usually cause increase of peroxidase activity, but contradictory results also exist, where decrease of peroxidase activity is observed during abiotic stress (van Doorn, Kesta 2014). A similar mechanism might have contributed to the decrease of peroxidase activity found in this study. We observed significant increase of phenolic concentration in the presence of elevated sucrose, which may be associated with increased polyphenol oxidase activity. During plant growth sucrose alters the C and N ratio in plants, thus coordinating many processes of plant development including maturation and synthesis of secondary metabolites (Zheng 2009). Phenolic synthesis requires carbohydrates, which explains the sharp increase in phenolic concentration in the presence of high sucrose observed in this experiment. A similar effect was also observed in *Camellia sinensis* plants *in vitro* (Qian et al. 2018). Therefore, sucrose might play a role in birch *in vitro*

shoot recalcitrance and lowered sucrose concentration might be beneficial during rejuvenation.

In conclusion, peroxidase and polyphenol oxidase activity, as well as total phenolic concentration can be used as indicators for birch *in vitro* shoot rejuvenation status, indicating increased peroxidase and polyphenol oxidase activity and higher phenolic concentration in mature recalcitrant shoots compared to rejuvenated shoots. *In vitro* factors, like prolonged subculture time, are not likely to cause maturation. However, high sucrose concentration might be involved in maturation, by enhancing phenolic synthesis and elevating polyphenol oxidase activity.

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