Identification of stress biomarkers for drought and increased soil temperature in seedlings of European beech (*Fagus sylvatica L.*)

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Abstract

Drought is an environmental stress that impacts plant productivity. Projections show both an increase in intense rain events and a reduction in the number of rain days, conditions that leads to increased risk of drought. Consequently, the identification of molecular biomarkers suitable for evaluating the impact of water deprivation conditions on forest plant seedlings is of significant value for monitoring purposes and forest management. In this study we evaluated a biochemical methodology for the assessment of drought stress coupled to variable soil temperature in European beech (*Fagus sylvatica* L.) seedlings by analyzing a set of metabolites and enzymes involved in free radical scavenging and cell wall synthesis. The results indicate that the specific activities and isoform profile of superoxide dismutases and glutathione peroxidases together with the variation of phenolic compounds enable discrimination between seedlings with different degree of photosynthetic activity. This approach represents a promising platform for the assessment of drought stress in forest trees and could serve for enhancing selection and breeding practices allowing for plants more tolerant of abiotic stress.

Keywords: beach; drought; superoxide dismutase; oxidative stress; glutathione peroxidase;

1. Introduction

Plants are exposed to different environmental stress factors that affect their growth and development. Drought is considered the single most critical environmental stress, which decreases plant productivity more than any other environmental stress (Reichstein et al. 2013; Running et al. 2004). From the plant perspective, drought is the deficit of moisture required for normal plant growth, development and completion of the life cycle (Manivannan et al. 2008a). Water deficit arises from insufficient available soil water because of reduced rainfall and elevated evaporation during the growing season . Climate projections show a reduction in the number of rainy days and an increase in evaporation in temperate areas due to elevated temperatures, a process that will cause increased risk of drought (Heyder et al. 2011; Trenberth 2011; Williams et al. 2013). The effects of drought are expected to be more intense in disturbed forest ecosystems (e.g. due to logging, forest fires, and bark beetle outbreaks) with reduced vegetation cover as deforestation increases ground surface temperature (Lewis 1998) in addition to general increase in temperature due to global change. As tree seedlings have shallow roots systems, the reforestation in such sites might be particularly hampered.

Drought increases the amplitude of oxidative stress in plant tissues, in which reactive oxygen species (ROS), such as superoxide radical, hydroxy radical, hydrogen peroxide and alkoxy radical are produced (Cruz de Carvalho 2008; Lewis 1998; Moran et al. 1994; Sharma and Dubey 2005). Superoxide radicals are rapidly dismutated by superoxide dismutase to hydrogen peroxide that can be eliminated by different enzymes such as peroxidases and catalases. These metalloenzymes constitute an important primary defense of cells against ROS and their activity is usually augmented during stress conditions (Manivannan et al. 2008b; Sharma and Dubey 2005).

European beech (*Fagus sylvatica*) is one of the most important deciduous trees in central European forests where it covers about 12 million hectares of land (Teissier du Cros et al. 1981). Changes in quantity and quality of beech wood could have a substantial ecological and economic impact. The identification of both qualitative and quantitative stress-related biomarkers suitable for validating drought stress models adapted to beech will be of outmost importance for forest industry and farming, as it has been in plant and human diagnostics (Carvajal-Hausdorf et al. 2015; Weber-Lotfi et al. 2005; Yin et al. 2016). Apart from industrial importance, sheer abundance of beech in temperate central European forest ecosystems makes this species critical to the overall forest health and ecosystem functioning.

In a previous work we explored the opportunity to use biochemical markers for monitoring temperature stress in European beech. The promising results prompted us to improve the methodologies for the identification of appropriate biomarkers for beech seedlings related to drought stress and drought stress in combination with increased soil temperatures. To achieve this, several biochemical parameters were compared to physical and physiological parameters.

2. Material and methods

2.1 Plant material

One-year old beech seedlings of provenance Osankarica 2.0119 (1240 m a.s.l., 46°27' N, 15°23' E) grown from seeds were obtained from the tree nursery Omorika d.o.o., Muta, N Slovenia, and planted in rhizotrons (one seedling per rhizotron) at Slovenian Forestry Institute in Ljubljana, central Slovenia on 22.3.2011. The external size of the rhizotrons measured 30x50x3 cm, while the internal size was 28x49x2 cm. The bottom third of the rhizotrons was filled with sand to allow for water draining, while the upper two thirds were filled with dystric cambisol originating from sandstone and slate ground rock collected from the upper soil horizon (0 to 30 cm) in a mixed forest in the vicinity of the Slovenian Forestry Institute. The soil was sieved through a 5x5 mm sieve, autoclaved and mixed with quartz sand, vermiculite and perlite in ratio 5:5:1:1. No fertilizer was used during the experiment.

The rhizotrons were transferred into a climatized room (IMP Klima, Godovič, Slovenia) set to 16 °C. Twelve seedlings were planted per rhizotron with the belowground portion protected from light, while 14 seedlings were placed in a cooled rhizoston the belowground part. Rhizothrons were arranged one by another in a way that between two consecutive seedlings there was one rhizotron filled only with soil to prevent intertwining of seedlings. Rhizothrons were placed vertically with slight inclination. The temperature of refrigerator was set to four degrees below the air temperature. The treatment with cooling of belowground part was used to reproduce the natural temperature gradient from aboveground to belowground in intact forests, while treatment without cooling of roots represented disturbed forest sites without protective vegetation cover and therefore increased soil temperatures. Soil temperatures for the treatment with cooled belowground were comparable with the maximum summer soil temperatures at a depth of 30 cm at the origin of the provenance of the seedlings used in this study.

Soil temperature in all treatments was monitored at -20 cm depth using factory calibrated digital temperature sensors DS18B20 connected to a datalogger developed at SFI, while air temperature and humidity were logged by USB dataloggers Voltcraft DL-120TH (Conrad Electronic, UK). A summary of the physical parameters during the seedling growth is reported in Table 1. Seedling were irrigated with automatic watering system with frequency domain soil moisture sensors EC-5 (Decagon Devices Inc., Pullman,USA). At 18.6.2014, the watering of half of the non-cooled seedlings and half of the cooled seedlings was stopped (the onset of drought experiment), resulting in four treatments: seedlings with cooled roots + water (CCRW+, 7 seedlings), seedlings with cooled roots – water (CCRW-, 7 seedlings), seedlings with non-cooled roots + water (NCRW+, 6 seedlings) and seedlings with non-cooled roots – water (NCRW-, 6 seedlings). Each treatment was replicated once. Just before drought treatment height of the seedlings was measured with tape meter and stem diameter two centimeters above the root neck recorded.

With the onset of drought experiment, soil moisture was monitored additionaly with the frequency domain sensor in every rhizothron twice per week and soil moisture in CCRW+ and NCRW+ treatments corrected by hand watering, when recording of soil moisture of watered seedlings on frequency domain sensor fell below 1000 mV (corresponding to soil matrix potential of approximately -0.15 MPa). For the soil used to fill the rhizotrons in our experiment, gravimetrical calibration of the EC-5 sensor in a cylinder of 0.8 dm^3 was done. At the same time, the soil matrix potential used in our experiment was measured with a MPS-1 sensor (Decagon Devices Inc.). Recalculation of the automatic irrigation system threshold provided true values of SWC and soil matrix potential used in our experiment. Intensity of photosynthetically active radiation just above the seedlings was around 300 µmol $m^{-2}s^{-1}$. Measurements of gas exchange, stomatal conductance, transpiration and photochemical efficiency of s-PSII were performed with Li-COR 6200 on 16.6. (2 days before onset of drought treatment), 8.7. (21 days after the onset of drought treatment), 23.7. (36 days after drought treatment) and 20.8.2014 (64 days after the onset of drought treatment), between 9-11 am. Measurements were performed on one fully developed leaf per plant from the middle of the crown at 400 µmol CO_2 mol^{-1}, photosynthetic photon flux density of 300 µmol m^{-2} s^{-1}. Leaf temperature varied according ambient temperature but was kept constant during measurement. Water vapour pressure deficit ranged from 0.70 to 1.30 kPa. Leaves were subsequently harvested and chlorophyll content measured with SPAD meter. Relative water content of leaves was measured on leaves used for chlorophyll measurements. Leaves were weighed, put onto moist filter paper and incubated for 24 hours at 5°C in the refrigerator. Turgid leaves were weighed again, dried and their dry weight recorded. From this data, leaf relative water content was calculated according to the following formula: RWC(l) (%) = (fresh – dry weight) / (turgid – dry weight) x 100. On 23.7. and 20.8.2014, additional three leaves per plant were randomly collected and frozen at -80ºC until further analysis. However, leaves collected on 20.8.2014 from drought-treated plants were too dry to recover material sufficient for a complete biochemical analysis. Since the major scope of the work was to identify early-stage biomarkers of drought stress, comparative biochemical measurements were performed only with the samples recovered in July .

2.2 Homogenization and extraction of plant material

Plant material used in the experiment was obtained by sampling the seedling in the 21st day after the onset of drought treatmen. Plant material used in the experiment was obtained by sampling the seedling in the 21st day after the onset of drought treatmen. Working temperature throughout the experiments was kept at 4ºC. Homogenization was performed on ice using mortar and pestle. Solvents used for homogenization were precooled overnight at 4°C . Prior to freezing, each individual leaf was weighed. Individual leaves were homogenized using 1 mL of appropriate solvent per 0.1 g of weighed material (Table 2). Frozen material was thawed during homogenization in a cooled extraction buffer. For analysis of enzymatic activity and protein content, plant material was extracted in 50 mM Tris-HCl buffer, pH 7.4, while 80% methanol in water was used for the analysis of phenolic compounds. Plant homogenate was rocked at 4^{o}C for 2 hours after which it was centrifuged 10 min at 14,000x*g* at 4°C. The supernatant was used for the experiments after determination of total protein concentration by using Quant-IT^{TM} protein assay (Life Technologies, USA) according to the manufacturer's instructions.

2.3 Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagent (Singleton and Rossi. 1965). Ten microliters of extract were mixed with 75 µL of 10-fold diluted Folin-Ciocalteu reagent and incubated 5 min at 22°C before the addition of 75 µL of sodium bicarbonate (0.72 M) solution. Absorbance was measured at 620 nm using a HTS7000 Bioassay reader (Perkin Elemer, USA) after 90 min of incubation at 22°C. Results are expressed as galic acid equivalents per mL of solution. Triplicate measurements were performed for each sample.

2.4 Measurement of peroxidase enzyme activities

Peroxidase activity (POX) was detected using o-dianisidine (Sigma-Aldrich, Germany) as a substrate (Pine, Hoffman. 1984). The reaction mixture was prepared by mixing 20 mL of 50 mM phosphate buffer, pH 7.0 with 9.79 mM hydrogen-peroxide and 0.2 mL o-dianisidine solution in methanol 11.1 mM. The reaction was initiated by adding plant extract (50 μL) to the reaction mixture (900 μL) in the measuring cuvette. After accurate stirring, the change in absorbance at 430 nm was read for 5 min at RT using a Perkin Elmer lambda 35 UV/Vis spectrophotometer (Perkin Elmer, USA). One unit of peroxidase activity was defined as the amount of the enzyme that oxidizes o-dianisidine into 1 μM of bis-(3,3′-dimethoxy-4-amino) azodiphenyl per min at 25ºC with the extinction coefficient 30 mM^{-1} cm^{-1}. Triplicate measurements were performed for each sample.

2.5 Measurement of superoxide dismutase enzyme activities

Total superoxide dismutase (SOD) activity was assayed by its ability to inhibit photochemical reduction of nitrobluetetrazolium (NBT, Serva, Germany) to blue formazan (Winterbourn et al. 1975). The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 0.66 mM EDTA, 10 mM $_L$-methionine, 33 μM NBT, and 3.3 μM riboflavin. The reaction was initiated by adding plant extract (50 μL) to the reaction mixture (200 μL). After mixing, samples were illuminated with sunlight for 10 min and absorbance at 492 nm was recorded using HTS7000 Bioassay reader (Perkin Elemer, USA). The blank was prepared by mixing extraction buffer with reaction mixture and kept in the dark while positive control was prepared in the identical manner and exposed to sun light same as the samples. One unit was defined as the amount of protein causing a 50% inhibition of NBT photoreduction. To determine the contribution of the single enzyme isoforms, 0.4% H_2O_2 was used for simultaneous inhibition of both forms Cu/ZnSOD and FeSOD, while 5 mM KCN was applied for specific Cu/ZnSOD inhibition (Sandalio et al. 1987). MnSOD activity was calculated as the residual activity after H_2O_2 inhibition. The H_2O_2- and KCN-sensitive activity was attributed to Cu/ZnSOD while FeSOD activity was inferred by subtracting Cu/ZnSOD activities from H_2O_2 -inhibited SOD activity. Triplicate measurements were performed for each sample.

2.6 Measurement of glutathione peroxidase enzyme activities

Glutathione peroxidase (GPX) activity was assessed by measuring the H_2O_2-dependent oxidation of glutathione GSH into GSSG (Wendel 1980). GSSG content was then determined in a coupled reaction in which glutathione reductase reduced the substrate into GSH oxidizing NADPH into NADP. The reaction mixture contained 48 mM sodium phosphate, pH 7.8, 0.38 mM EDTA, 0.12 mM NADPH, 3.2 U of glutathione reductase, 1 mM GSH, 0.02 mM $_{DL}$-dithiotritol, and 2.28 mM H_2O_2. The rate of NADPH oxidation measured at 340 nm for 3 min was recorded using a HTS7000 Bioassay reader (Perkin Elemer, USA). One unit is defined as the amount of protein able to catalyze the oxidation of 1 μM of GSH per min at pH 7.0 and RT. Triplicate measurements were performed for each sample.

2.7 Native PAGE

For in-gel analysis of enzymatic activity, aqueous plant extracts were resolved under non-reducing conditions in a discontinuous buffer system using a vertical electrophoresis slab system (Hoefer, Holliston, USA) with a 4 % (w/v) stacking and a 10 % (w/v) resolving gel (Table 2). Each gel lane was loaded with 12.5 μg of total protein. POX activity was identified according to a modified Quesada protocol (Quesadaet.al. 1990). After electrophoresis, the gel was washed twice with 50 mM acetate, pH 6.0, after which it was incubated 1 hour in 50 mM acetate, pH 6.0, 28 mM o-dianisidine, and 36.4 mM H_2O_2.

2.8 Statistical analyses

Statistical analysis was performed using GraphPad Prism v5.03 for Windows (San Diego, California, USA). A significance level of $p \leq 0.05$ was used for analysis of variance, implemented using the Kruskal-

Wallis test followed by the Tukey's post-hoc test ($p \leq 0.05$). Correlation between different parameters was performed at significance level of $p \leq 0.1$. Unsupervised hierarchical clustering and heatmap generation was accomplished using ClustalVis (Metsalu and Vilo 2015) with the Manhattan method and Pearson correlation for the distance measure. For heatmap generation, measured values were standardized across the two cohorts by conversion to Z-scores (peak height-mean/standard deviation).

3 Results

3.1 Effect of growth parameters on physiological parameters

The experimental growth conditions were chosen to reproduce the natural temperature gradient from aboveground to belowground in intact (cooling of belowground part: CCRW) and disturbed forest sites without protective vegetation cover and consequently increased soil temperatures (NCRW).

Height of seedlings grown at control growth conditions (CCRW) was 37.0 ± 1.91 cm, and 27.7 ± 2.16 for seedlings grown at increased soil temperatures (NCRW). Stem diameter was 6.25 ±0.30 mm for CCRW seedlings and 5.58 ± 0.14 mm for NCRW seedlings. Similarly, reduced stem diameter, as well as reduced root and shoot biomass, was detected in beech seedlings grown at increased soil temperature in experiment with the same beech provenance and growth conditions conducted by Štraus et al. (2014), indicating the limiting effect of increased soil temperatures on growth.

In present experiment, the combined effects of drought and soil temperatures were tested. Soil matrix potential of non-watered seedlings decreased rapidly after the onset of drought treatments, regardless of soil temperature. Visually, drought symptoms were observed as yellowing and drying of smaller leaves, first symptoms were noticed at around -0.5 MPa in both groups 17 days after the onset of drought. However, for fully developed leaves relative leaf water contents between treatments were, similarly to pre-treatment measurement, not significantly different between each other 21 days after the onset of experiment (Kruskal-Wallis test, H=5.5881, p=0.1335), although there was a decreasing tendency for NCRW- already observed (Fig. 1). Thirty-six days after the onset of the experiment, relative water content of NCRW- treatment (less than 20 %) was significantly different from both watered treatments, CCRW+ and NCRW+ (Kruskal-Wallis test, H=15.6931, p=0.0013). Slightly reduced values of relative water content (around 60%) were observed also for CCRW- treatment. Relative water contents of leaves for both drought treatments have further decreased in the last sampling, 64 days after the onset of drought treatment.

One month after the onset of drought, soil matrix potential decreased below -0.6 MPa and stayed at around -0.7 MPa until the end of the experiment (Fig. 2a). Zang et al. (2014), regarded soil matrix potential of -0.4 MPa as moderate drought for beech seedlings in rhizotrons, while less than -1.0 MPa was assigned as severe drought stress where irreversible damage occurs. Both, net photosynthesis and stomatal conductance correlated well with soil matrix potential (Fig. 2b, c).

Net photosynthesis levels were very low in all treatments (Fig. 2b), with the maximum of 5.25 µmol $CO_2 m^{-2} s^{-1}$ achieved in the watered seedlings with non-cooled roots (NCRW+). Seedlings with non-cooled roots exposed to drought stress (NCRW-) exhibited levels of net photosynthesis below zero already at day 21 after the onset of drought treatment (soil matrix potential – 0.70 MPa, Figs. 3b), while positive net photosynthesis was still observed after 36 days in seedlings with cooled roots exposed to drought (CCRW, soil matrix potential -0.65MPa, Figs. 3b). At day 64 we were unable to measure gas exchange in both drought treatments (Fig. 3a-c), with soil matrix potential < -0.75MPa (Fig. 2a). Altogether, our results show that under the designed experimental conditions the variations of soil matrix potential were well mirrored in net photosynthesis (Fig. 2b) and stomatal conductance (Fig. 2c). Negative net photosynthesis in non-cooled seedlings exposed to drought was accompanied by very low stomatal conductance (Fig. 3b), but high intercellular CO_2 concentration (Fig. 3c), indicating severely reduced ability of photosynthetic apparatus to assimilate CO_2, although chlorophyll levels were still relatively high (Fig. 3d). In this condition respiration significantly exceeded photosynthesis (Fig. 3a). Thirty-six days after the onset of drought, seedlings with cooled roots still maintained internal CO_2 concentrations comparable with watered treatments (Fig. 3c), although stomatal conductance was significantly lower than in both watered treatments (Fig. 3b). Assuming the approximation that net photosynthesis inversely correlates with plant stress, the data confirm that higher soil temperatures can be relatively well tolerated in the presence of water availability (NCRW+), but substantially increase the stress conditions when water becomes limiting (NCRW-).

3.2 Effects of drought on total phenolic content and enzyme activity

The minimal total phenolic content was determined in the fittest sample (NCRW+) whereas it was three times lower than in all the others (Fig. 4, Fig 8.). This result indicates that total phenolic content is a valuable biomarker for assessing photosynthetic stress, as recently demonstrated for salt-induced plant stress (Aloisi et al. 2016), although it does not discriminate between drought and conditions characterized by low soil temperature.

Next we measured the activity of some key enzymes involved in the scavenging of active oxygen forms. Peroxidase (POX) activity (Fig. 5a, Fig 8.) did not seem to be influenced by drought stress. Only the total activity of the CCRW+ sample significantly increased. The analysis of the different isoforms in the zymograms revealed an extremely complex pattern (Fig. 5b, Fig 8.). Since POXs are also involved in other metabolic activities, conclusions could be inferred only after a precise and time consuming characterization of each isoform. Therefore, despite their high stability and the simple measurement of their activity, in the case of drought-effect evaluation POXs do not fulfil the requisites of clarity and measurement simplicity that a biomarker should possess.

In this context, SOD appeared as an extremely more attractive candidate for discriminating among stress conditions characterized by different level of photosynthetic capacity. Total SOD activity of NCRW+ was the lowest of the four samples (Fig. 6a, Fig 8.) and other interesting discriminating parameters are offered by the specific activities of the single SOD isoforms. MnSOD and FeSOD specific activities of NCRW+ were half of that measured in the other samples, whereas the same seedlings showed negligible differences in Cu/ZnSOD activity in comparison to the other samples (Fig. 6b, Fig 8.).

Similarly to FeSOD, also glutathione peroxidase activity seems a very useful biomarker of drought stress. It directly correlated with the seedling photosynthetic activity, being maximal in NCRW+ and significantly higher also in CCRW+ in comparison to drought-stressed samples (Fig. 7, Fig 8.).

4 Discussion

Plants, as sedentary organisms, have to adapt their physiology to environmental changes in order to survive and thrive. Out of all environmental stressors, drought has the most detrimental impact on plant growth, productivity and survival (Pompelli et al. 2010). Under drought stress, photosynthesis decreases due to stomatal limitation when light energy absorption exceeds its capacity for utilization (Cornic 2000). The excess light energy, which is neither consumed in photosynthesis nor dissipated as fluorescence or heat, is transferred to oxygen or neighboring molecules, creating ROS, including superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl free radical (Silva et al. 2010). This causes oxidative damage to cellular components and structures and disrupts metabolism, which finally leads to cell death (Silva et al. 2010). Alleviation of oxidative stress relies on combination of both enzymatic and non-enzymatic systems and there are numerous reports dealing with changes in antioxidative factors in response to drought stress (Liu et al. 2014; Marabottini et al. 2001; Uzilday et al. 2012).

In this study we aimed at using a set of simple biochemical tests to evaluate the possibility of their application as early markers of drought in the industrially important and highly abundant European beech. The most important quality of a biomarker is its ability to clearly discriminate between stressed and control samples. Furthermore, its recovery, processing, and analysis should be simple, reliable, and allow for large-scale data comparison among samples collected under variable field conditions. Enzymes of antioxidative defense are logical choice since their activity changes during many physiological and pathological processes (Cruz de Carvalho 2008; De Marco and Roubelakis-Angelakis 1996; Jenks and Hasegawa 2005; Lipiec et al. 2013) in plants. Peroxidases fit the desired biomarker criteria as they are easy to detect, process and analyze (Running et al. 2004) but are not suitable for describing the specific stress conditions tested in the present experiments. Probably a further effort would be necessary to identify the isoforms specifically involved in drought stress to distinguish them from those playing complementary physiological roles. In our case, we cannot rule out that the experimentally imposed growth conditions induced additional stresses to the drought. For instance, seedlings with cooled roots could be subjected to nutrient shortage as they were grown for several years without addition of nitrogen and it is well known that nitrogen has lower availability at lower soil temperatures due to lower nitrogen mineralization (Zhou et al. 2011). This could explain the experimental deviation of some measured parameters between NCRW+ and CCRW+ samples. In any case, we identified some biochemical markers able to discriminate between seedlings with elevated and scarce photosynthetic activity. The most selective changes induced by drought and temperature affected the activity of FeSOD and GPX, whereas other biomarkers monitored broader stress conditions. MnSOD could be potentially a very useful marker in conditions where drought stress is combined with higher soil temperature.

Similarly to what recently observed in salt- and cadmium-stressed plants , phenolic content significantly increased in drought-treated samples and in seedlings with sufficient water supply but constantly cooled roots. As phenolics in trees increase due to drought stress ((Grulke and Tausz 2014)) and decrease due to elevated temperatures (Zvereva and Kozlov 2006) this is a logical outcome. In contrast, glutathione peroxidase activity was strongly and selectively decreased only in drought-stressed seedlings with respect to those in which photosynthesis was active due to water availability. The high glutathione peroxidase activity in these plants would suggest the necessity to cope with ROS in photosynthetically active cells. SOD isoforms present in the seedlings represent another group of highly informative biomarkers. Their expression and activity is regulated by different stress factors (Grulke and Tausz 2014) and in the case of beech seedlings it seems that stress conditions strongly activated the mitochondrion-specific MnSODs as well as the (putative) chloroplast-specific FeSODs, whereas the Cu/ZnSOD activity was not significantly challenged. The physiological explanation of these changes is beyond the aim of this work, but the hypothesis of oxidative stress in chloroplasts subject to conditions of limiting water supply and in mitochondria over-activated due to the lack of other suitable cellular energy sources seems plausible. It has been already reported that active oxygen unbalance caused by methyl viologen activated the expression of new FeSOD isoforms in the chloroplasts (Zvereva and Kozlov 2006) and that effective MnSOD activity in the mitochondria protected rice from drought-induced stress (Prakash et al. 2016). The constant Cu/ZnSOD activity in all the samples would rather correspond to housekeeping activities such as cell-wall synthesis (Kim et al. 2008). It will be meaningful to confirm the apparent high inverse correlation between FeSOD and photosynthetic activity by measuring the expression variation of other proteins involved in FeSOD activity, as for instance chaperonin 20 (Kuo et al. 2013).

The statistical analysis indicates that both enzyme biomarkers and phenolic compounds can be effectively exploited to discriminate between seedlings grown under different conditions. The dendrogram and accompanying heat map further depict the relative incidence of such biomarkers in the analyzed samples and graphically indicate that the observation of biomarker combinations is more informative than the quantification of single biomarkers (Fig 8).

The collected results clearly indicate the feasibility of using a set of biomarkers as a tool for evaluating the physiological condition of sampled plants thus enabling insight into the overall health conditions of a forest region and the impact of environmental stressors on it. Furthermore, coupling the variation of more biochemical parameters could represent a simple and reproducible monitoring method for detection of early signs of water deprivation. This approach developed for seedlings could be a promising platform for the evaluation of drought stress in adult forest plants in combination with orthogonal techniques such as genomic and proteomic analyses. We consider the analyses of other biomarkers for sensing milder stress conditions, as contained temperature variations. For their nature, heat shock proteins seem appropriate candidates for this application, as already proved for crops and proposed for studying heat acclimation (Driedonks et al. 2015; Jacob et al. 2016). Establishment of this type of monitoring could contribute in improving the selection and breeding practices as they would simplify the identification of more resilient and adaptable clones.

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6 Compliance with ethical standards

The authors declare that they have no conflict of interest. This research did not involve any Human participants and/or Animals. All the authors have made a significant contribution to this manuscript, have seen and approved the final manuscript, and have agreed to its submission to the Canadian Journal of Forest Research.

7 References

Bollag, D.M., Rozycki, M.D., and Stuart, J.E.n.e. 1996 Protein Methods. Wiley-Liss, New York.

Carvajal-Hausdorf, D.E., Schalper, K.A., Neumeister, V.M., and Rimm, D.L. 2015. Quantitative measurement of cancer tissue biomarkers in the lab and in the clinic. Lab. Invest. **95**(4): 385-396. doi: 10.1038/labinvest.2014.157.

Cornic, G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture – not by affecting ATP synthesis. Trends Plant Sci. **5**(5): 187-188. doi: 10.1016/s1360-1385(00)01625-3.

Cruz de Carvalho, M.H. 2008. Drought stress and reactive oxygen species: Production, scavenging and signaling. Plant Signaling & Behavior **3**(3): 156-165.

De Marco, A., and Roubelakis-Angelakis, K.A. 1996. The Complexity of Enzymic Control of Hydrogen Peroxide Concentration May Affect the Regeneration Potential of Plant Protoplasts. Plant Physiol. **110**(1): 137-145. doi: 110/1/137 [pii].

Driedonks, N., Xu, J., Peters, J.L., Park, S., and Rieu, I. 2015. Multi-Level Interactions Between Heat Shock Factors, Heat Shock Proteins, and the Redox System Regulate Acclimation to Heat. Front Plant Sci **6**: 999. doi: 10.3389/fpls.2015.00999.

Grulke, N., and Tausz, M. 2014. The Future of Trees in a Changing Climate: Synopsis. *In* Trees in a Changing Environment: Ecophysiology, Adaptation, and Future Survival. *Edited by* M. Tausz and N. Grulke. Springer Netherlands, Dordrecht. pp. 265-279.

Heyder, U., Schaphoff, S., Gerten, D., and Lucht, W. 2011. Risk of severe climate change impact on the terrestrial biosphere. Environmental Research Letters **6**(3): 034036.

Jacob, P., Hirt, H., and Bendahmane, A. 2016. The heat shock protein/chaperone network and multiple stress resistance. Plant Biotechnol J. doi: 10.1111/pbi.12659.

Jenks, M., and Hasegawa, P. 2005. Plant Abiotic Stress. Blackwell Publishing, Oxford

Kim, H.J., Kato, N., Kim, S., and Triplett, B. 2008. Cu/Zn superoxide dismutases in developing cotton fibers: evidence for an extracellular form. Planta **228**(2): 281-292. doi: 10.1007/s00425-008-0734-0.

Kuo, W.Y., Huang, C.H., Liu, A.C., Cheng, C.P., Li, S.H., Chang, W.C., Weiss, C., Azem, A., and Jinn, T.L. 2013. CHAPERONIN 20 mediates iron superoxide dismutase (FeSOD) activity independent of its co-chaperonin role in Arabidopsis chloroplasts. New Phytol **197**(1): 99-110. doi: 10.1111/j.1469-8137.2012.04369.x.

Lewis, T. 1998. The effect of deforestation on ground surface temperatures. Global Planet. Change **18**(1–2): 1-13. doi: http://dx.doi.org/10.1016/S0921-8181(97)00011-8.

Lipiec, J., Doussan, C., Nosalewicz, A., and Kondracka, K. 2013. Effect of drought and heat stresses on plant growth and yield: a review. *In* International Agrophysics. p. 463.

Liu, C., Wang, Y., Pan, K., Li, W., Zhang, L., Shen, X., Liu, L., and Deng, M. 2014. Responses of the antioxidant defense system to drought stress in the leaves of Fargesia denudata seedlings, the staple food of the giant panda. Russian Journal of Plant Physiology **61**(3): 374-383. doi: 10.1134/s1021443714020083.

Manivannan, P., Jaleel, C.A., Kishorekumar, A., Sankar, B., Somasundaram, R., and Panneerselvam, R. 2008a. Protection of Vigna unguiculata (L.) Walp. plants from salt stress by paclobutrazol. Colloids Surf B Biointerfaces **61**(2): 315-318. doi: S0927-7765(07)00366-9 [pii] 10.1016/j.colsurfb.2007.09.007.

Manivannan, P., Jaleel, C.A., Somasundaram, R., and Panneerselvam, R. 2008b. Osmoregulation and antioxidant metabolism in drought-stressed Helianthus annuus under triadimefon drenching. C. R. Biol. **331**(6): 418-425. doi: http://dx.doi.org/10.1016/j.crvi.2008.03.003.

Marabottini, R., Schraml, C., Paolacci, A.R., Sorgona, A., Raschi, A., Rennenberg, H., and Badiani, M. 2001. Foliar antioxidant status of adult Mediterranean oak species (Quercus ilex L. and Q. pubescens Willd.) exposed to permanent CO2-enrichment and to seasonal water stress. Environ. Pollut. **115**(3): 413-423. doi: http://dx.doi.org/10.1016/S0269-7491(01)00231-7.

Metsalu, T., and Vilo, J. 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res. **43**(W1): W566-W570. doi: 10.1093/nar/gkv468.

Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V., and Aparicio-Tejo, P. 1994. Drought induces oxidative stress in pea plants. Planta **194**(3): 346-352. doi: 10.1007/bf00197534.

Pompelli, M.F., Barata-Luís, R., Vitorino, H.S., Gonçalves, E.R., Rolim, E.V., Santos, M.G., Almeida-Cortez, J.S., Ferreira, V.M., Lemos, E.E., and Endres, L. 2010. Photosynthesis, photoprotection and antioxidant activity of purging nut under drought deficit and recovery. Biomass Bioenergy **34**(8): 1207-1215. doi: http://dx.doi.org/10.1016/j.biombioe.2010.03.011.

Popović, M., Šuštar, V., Gričar, J., Štraus, I., Torkar, G., Kraigher, H., and de Marco, A. 2015. Identification of environmental stress biomarkers in seedlings of European beech (Fagus sylvatica) and Scots pine (Pinus sylvestris). Canadian Journal of Forest Research **46**(1): 58-66. doi: 10.1139/cjfr-2015-0274.

Prakash, C., Mithra, S.V.A., Singh, P.K., Mohapatra, T., and Singh, N.K. 2016. Unraveling the molecular basis of oxidative stress management in a drought tolerant rice genotype Nagina 22. BMC Genomics **17**(1): 774. doi: 10.1186/s12864-016-3131-2.

Reichstein, M., Bahn, M., Ciais, P., Frank, D., Mahecha, M.D., Seneviratne, S.I., Zscheischler, J., Beer, C., Buchmann, N., Frank, D.C., Papale, D., Rammig, A., Smith, P., Thonicke, K., van der Velde, M., Vicca, S., Walz, A., and Wattenbach, M. 2013. Climate extremes and the carbon cycle. Nature **500**(7462): 287-295. doi: 10.1038/nature12350.

Running, S.W., Nemani, R.R., Heinsch, F.A., Zhao, M., Reeves, M., and Hashimoto, H. 2004. A Continuous Satellite-Derived Measure of Global Terrestrial Primary Production. Bioscience **54**(6): 547-560. doi: 10.1641/0006-3568(2004)054[0547:ACSMOG]2.0.CO;2.

Sandalio, L.M., Palma, J.M., and Del Rio, L.A. 1987. Localization of manganese superoxide dismutase in peroxisomes isolated from Pisum sativum L. Plant Sci. **51**(1): 1-8. doi: http://dx.doi.org/10.1016/0168-9452(87)90213-5.

Sharma, P., and Dubey, R.S. 2005. Drought Induces Oxidative Stress and Enhances the Activities of Antioxidant Enzymes in Growing Rice Seedlings. Plant Growth Regulation **46**(3): 209-221. doi: 10.1007/s10725-005-0002-2.

Silva, E.N., Ferreira-Silva, S.L., Fontenele Ade, V., Ribeiro, R.V., Viegas, R.A., and Silveira, J.A. 2010. Photosynthetic changes and protective mechanisms against oxidative damage subjected to isolated and combined drought and heat stresses in Jatropha curcas plants. J Plant Physiol **167**(14): 1157-1164. doi: S0176-1617(10)00191-4 [pii]
10.1016/j.jplph.2010.03.005.

Štraus, I., Mrak, T., Ferlan, M., Železnik, P., and Kraigher, H. 2014. Influence of soil temperature on growth traits of European beech seedlings. Canadian Journal of Forest Research **45**(3): 246-251. doi: 10.1139/cjfr-2014-0332.

Teissier du Cros, E., Le Tacon, F., Nepveu, G.P., J., and Timbal, J.E. 1981. Le Hêtre. Département des Recherches Forestières, INRA, Paris.

Trenberth, K.E. 2011. Changes in precipitation with climate change. Clim. Res. **47**(1-2): 123-138.

Uzilday, B., Turkan, I., Sekmen, A.H., Ozgur, R., and Karakaya, H.C. 2012. Comparison of ROS formation and antioxidant enzymes in Cleome gynandra (C(4)) and Cleome spinosa (C(3)) under drought stress. Plant Sci **182**: 59-70. doi: S0168-9452(11)00092-6 [pii]
10.1016/j.plantsci.2011.03.015 [doi].

Weber-Lotfi, F., Obrecht-Pflumio, S., Guillemaut, P., Kleinpeter, J., and Dietrich, A. 2005. Specific plant DNA adducts as molecular biomarkers of genotoxic atmospheric environments. Mutat. Res. **581**(1-2): 55-67. doi: 10.1016/j.mrgentox.2004.10.020.

Wendel, A. 1980. Enzymatic Basis of Detoxication. Academic Press, NY. p. 333.

Williams, A.P., Allen, C.D., Macalady, A.K., Griffin, D., Woodhouse, C.A., Meko, D.M., Swetnam, T.W., Rauscher, S.A., Seager, R., Grissino-Mayer, H.D., Dean, J.S., Cook, E.R., Gangodagamage, C., Cai, M., and McDowell, N.G. 2013. Temperature as a potent driver of regional forest drought stress and tree mortality. Nature Clim. Change **3**(3): 292-297. doi: http://www.nature.com/nclimate/journal/v3/n3/abs/nclimate1693.html#supplementary-information.

Winterbourn, C.C., Hawkins, R.E., Brian, M., and Carrell, R.W. 1975. The estimation of red cell superoxide dismutase activity. J. Lab. Clin. Med. **85**(2): 337-341. doi: 0022-2143(75)90439-4 [pii].

Yin, C., Luo, C., Hu, W., Ding, X., Yuan, C., and Wang, F. 2016. Quantitative and Qualitative Analysis of Circulating Cell-Free DNA Can Be Used as an Adjuvant Tool for Prostate Cancer Screening: A Meta-Analysis. **2016**: 3825819.

Zang, U., Goisser, M., Häberle, K.-H., Matyssek, R., Matzner, E., and Borken, W. 2014. Effects of drought stress on photosynthesis, rhizosphere respiration, and fine-root characteristics of beech saplings: A rhizotron field study. J. Plant Nutr. Soil Sci. **177**(2): 168-177. doi: 10.1002/jpln.201300196.

Zvereva, E.L., and Kozlov, M.V. 2006. Consequences of simultaneous elevation of carbon dioxide and temperature for plant–herbivore interactions: a metaanalysis. Global Change Biol. **12**(1): 27-41. doi: 10.1111/j.1365-2486.2005.01086.x.

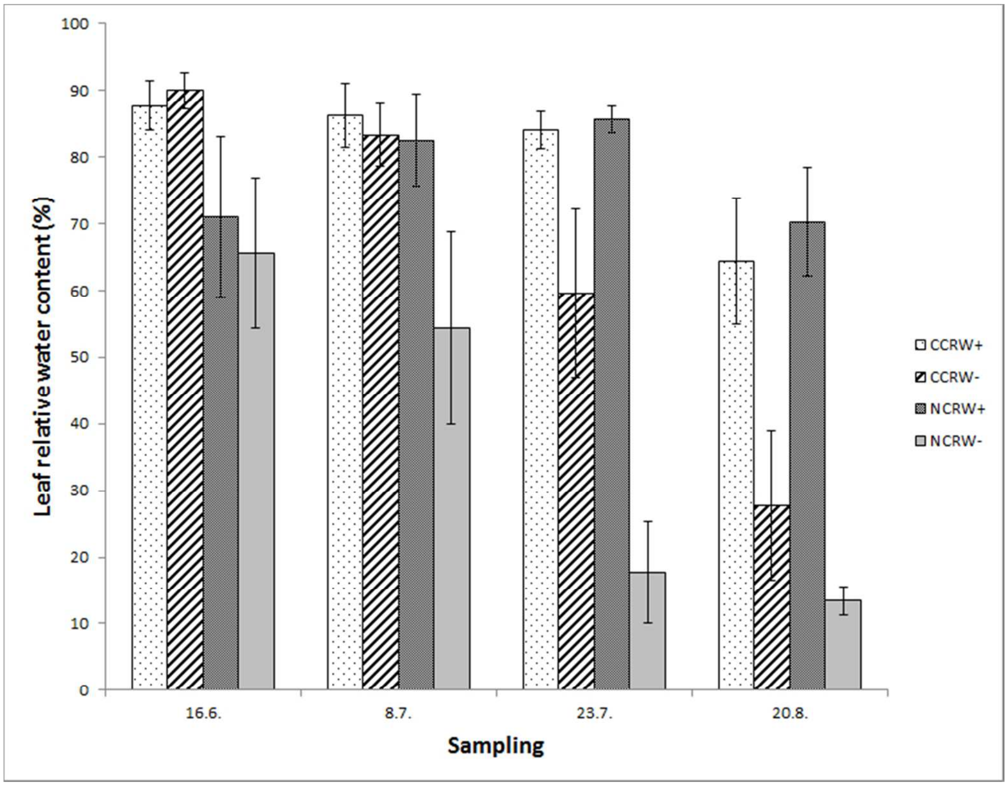

Figure 1: Leaf relative water content (%) of beech seedlings for treatments: NCRW+- Samples grown at 16°C with watering; CCRW-- Samples grown at 16°C with roots cooled to 12°C withouth watering; NCRW-- Samples grown at 16°C without watering. Sampling on 16.6.2014 was performed two days before the onset of drought experiment. Samplings on 8.7., 23.7. and 20.8.2014 were performed 21, 36 and 64 days after the onset of drought experiment, respectively.

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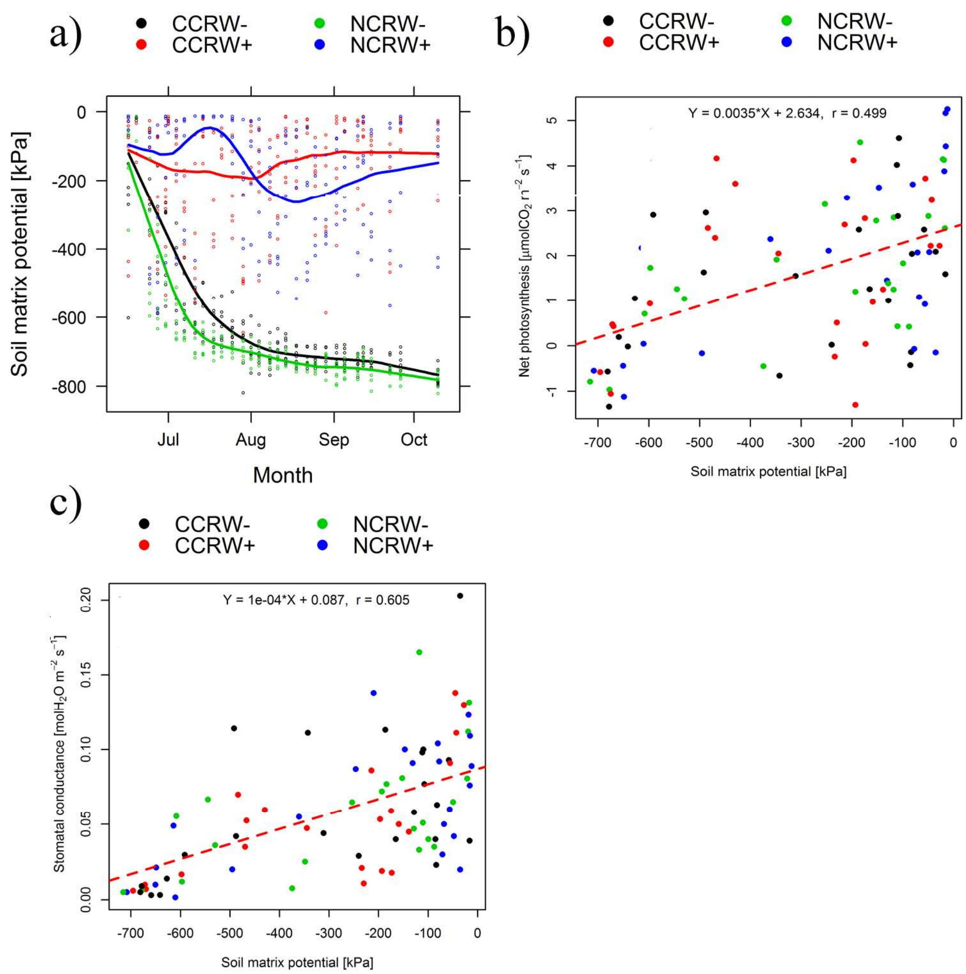

Figure 2. Time series of soil matrix potential in beech seedlings subjected to different soil temperatures and drought (A). Correlation between neto photosynthesis and soil matrix potential in beech seedlings subjected to different soil temperatures and drought, p=0.0000 (B). Correlation between stomatal conductance and soil matrix potential in beech seedlings subjected to different soil temperatures and drought, p=0.0000. (C) NCRW+- Samples grown at 16°C with watering; CCRW-- Samples grown at 16°C with roots cooled to 12°C without watering; NCRW-- Samples grown at 16°C without watering

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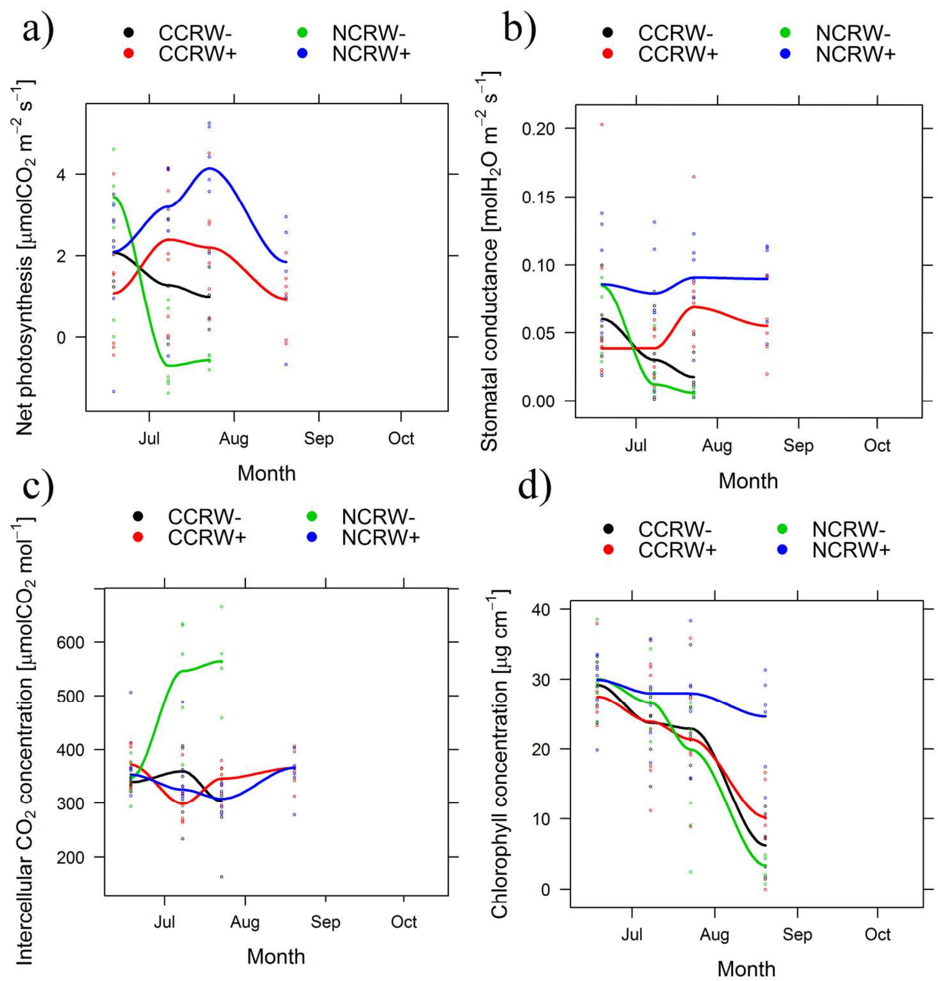

Figure 3. Time series of physiological parameters in beech seedlings subjected to different soil temperatures and drought: CCRW+- Samples grown at 16oC with roots cooled to 12oC with watering; NCRW+- Samples grown at 16°C with watering; CCRW-- Samples grown at 16°C with roots cooled to 12°C withouth watering; NCRW-- Samples grown at 16°C without watering.

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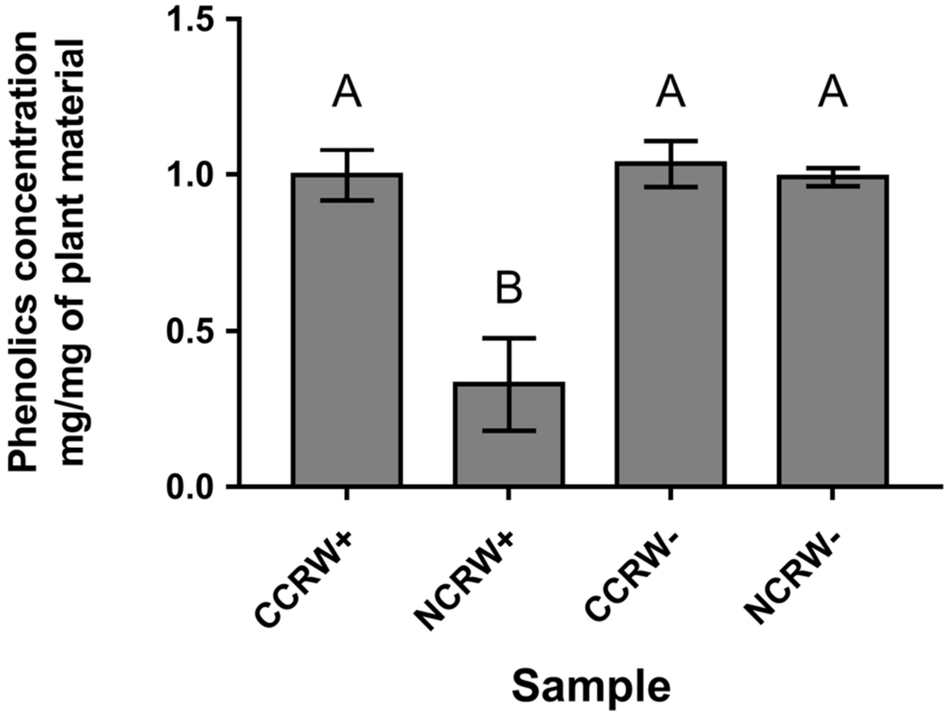

Figure. 4. Determination of total phenolic content of plant methanol extracts: Total phenolic concentration in plant methanol extracts expressed as galic acid equivalents: CCRW+- Samples grown at 16oC with roots cooled to 12oC with watering; NCRW+- Samples grown at 16°C with watering; CCRW-- Samples grown at 16°C with roots cooled to 12°C without watering; NCRW-- Samples grown at 16°C without watering. The errors bars indicate standard deviations for triplicate measurements. Means with different letters are significantly different (Turkeys HSD, $p \leq 0.05$)

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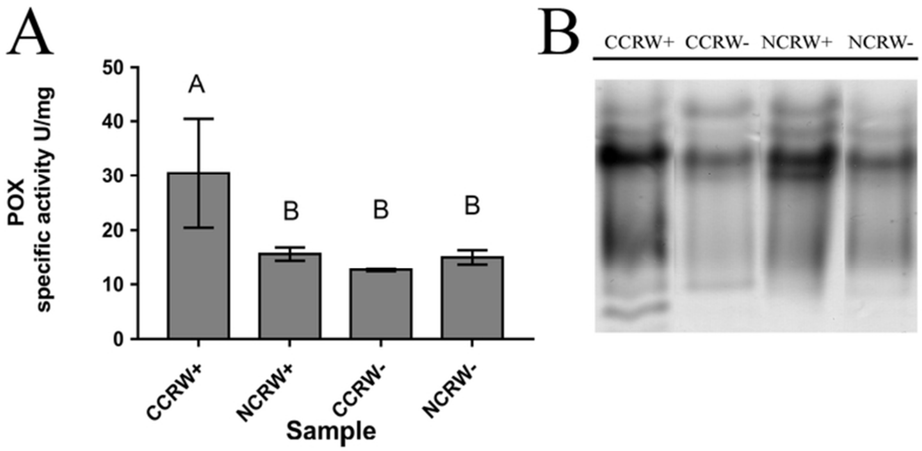

Figure. 5. Specific enzymatic activities of peroxidase in plant extracts (A); Electrophoretic separation of peroxidase isoforms (B): CCRW+- Samples grown at 16oC with roots cooled to 12oC with watering; NCRW+- Samples grown at 16°C with watering; CCRW-- Samples grown at 16°C with roots cooled to 12°C without watering; NCRW-- Samples grown at 16°C without watering. The errors bars indicate standard deviations for triplicate measurements. Means with different letters are significantly different (Turkeys HSD, $p \leq 0.05$)

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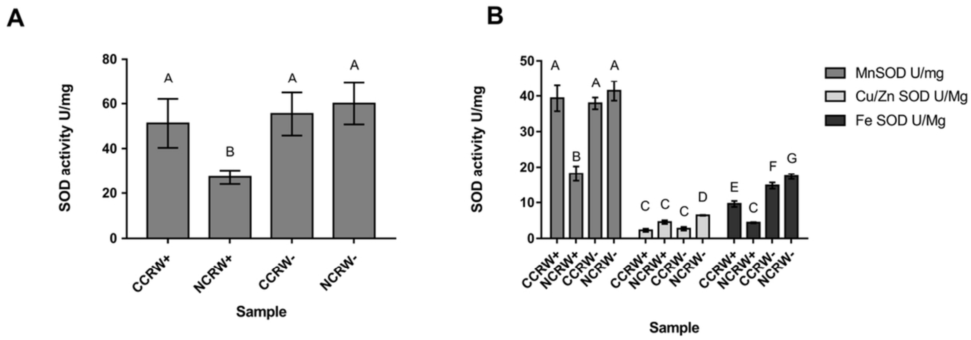

Figure. 6. Total specific enzymatic activities of superoxide dismutase (A) and specific activity of different SOD isoforms (B) in plant extracts: CCRW+- Samples grown at 16oC with roots cooled to 12oC with watering; NCRW+- Samples grown at 16^{o}C with watering; CCRW-- Samples grown at 16^{o}C with roots cooled to 12^{o}C without watering; NCRW-- Samples grown at 16^{o}C without watering. The errors bars indicate standard deviations for triplicate measurements. Means with different letters are significantly different (Turkeys HSD, $p \leq 0.05$)

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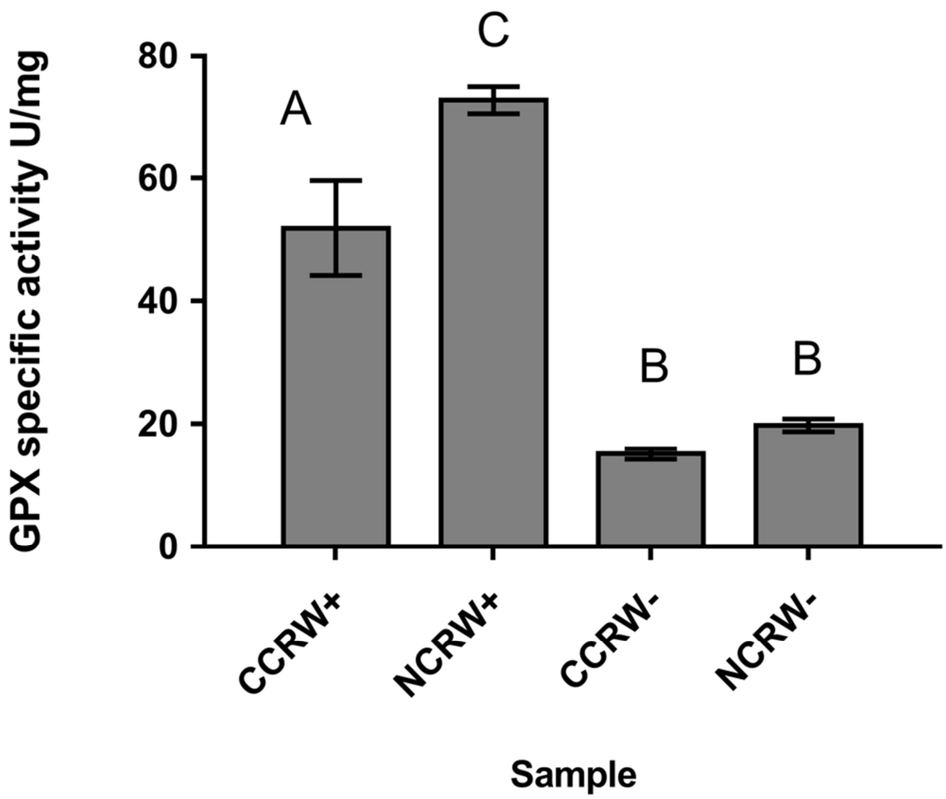

Figure 7. Specific enzymatic activities of glutathion peroxidase in plant extracts: CCRW+- Samples grown at 16oC with roots cooled to 12oC with watering; NCRW+- Samples grown at 16°C with watering; CCRW-- Samples grown at 16°C with roots cooled to 12°C without watering; NCRW-- Samples grown at 16°C without watering. The errors bars indicate standard deviations for triplicate measurements. Means with different letters are significantly different (Turkeys HSD, $p \leq 0.05$)

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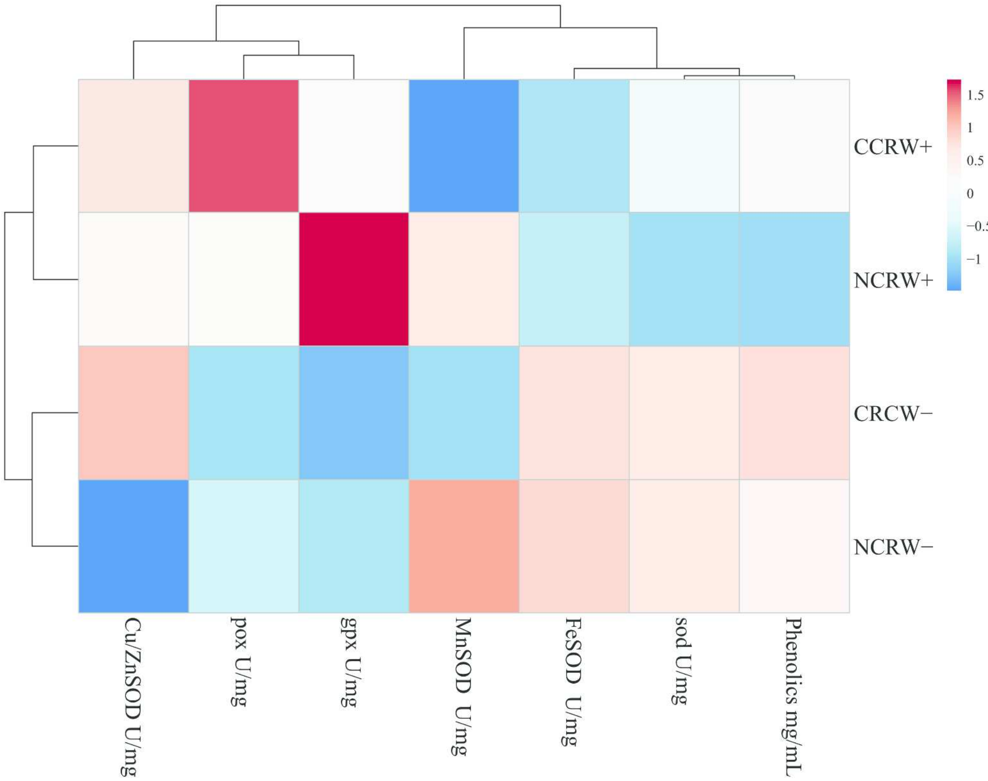

Figure 8. Heat map showing unsupervised hierarchical clustering of different biomarker values according to the seedling growth conditions: Measured biomarkers for beech seedlings are arranged in colums while growth coditions are in rows. Shades of red represent elevation of a metabolite while shades of blue represent decrease of a biomarker value, relative to the median levels (see color scale). In the dendrograms, the clustering clearly differentiates the stressed and control samples. Total phenolic concentration in plant methanol extracts is expressed in galic acid equivavelnts mg/ml (phenolics, mg/ml);. Specific enzymatic activities of peroxidase (POX, U/mg), superoxide dismutase (SOD, U/mg), glutathione peroxidase (GPX, U/mg) are represented in U/mg of total protein.

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Table 1: Seedling growth experimental conditions

CCRW$^+$- Samples grown at 16^{o}C with roots cooled to 12^{o}C with watering; NCRW$^+$- Samples grown at 16^{o}C with watering; CCRW$^-$- Samples grown at 16^{o}C with roots cooled to 12^{o}C withouth watering; NCRW$^-$- Samples grown at 16^{o}C withouth watering. Means are based on values recorded daily and every half an hour

	Air temperature (°C)			**Air RH (%)**			**CO_2 concentration (ppm)**			**Soil temperature at -20 cm depth (°C)**			**Water**
	Mean ± SD	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**	
NCRW$^+$ **NCRW$^-$**	16.0 ± 2.3	13.4	30.1	77.3 ± 5.6	0.5	90.9	671 ± 214	445	1548	15.1 ± 2.5	10.4	25.8	+ -
CCRW$^+$ **CCRW$^-$**	16.0 ± 2.3	13.4	30.1	77.3 ± 5.6	0.5	90.9	671 ± 214	445	1548	12.0 ± 2.9	9.1	25.9	+ -

Tree Species	**European beech (*Fagus sylvatica* L.)**	
Extraction procedure	50 mM Tris-HCl buffer, pH 7.4	80% methanol
Biomarker assessment	Enzyme specific activity (U/mg): POX, SOD, GPX Enzyme isoform distribution (Native PAGE): POX	Phenolic concentration (µg/mL)

Table 2: Experimental set-up

POX: peroxidase, SOD: superoxide dismutase, GPX: glutathione peroxidase,