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1 **Identification of environmental stress biomarkers in seedlings of European beech (*Fagus*
2 *sylvatica L.*) and Scots pine (*Pinus sylvestris L.*)**
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62
63
64 **Abstract**
65
66 Climate development models predict alterations that will critically influence plant metabolism in Southern
67 and Central Europe. Although the molecular players involved in the response to climatic stress factors
68 have been well described in crops, little information is available for forest tree species. Consequently, the
69 identification of molecular biomarkers suitable for evaluating the actual impact of different environmental
70 stress conditions on forest plants would be of great importance for monitoring purposes and forest
71 management. In this study we evaluated a biochemical methodology for the assessment of temperature
72 stress in European beech (*Fagus sylvatica* L.) and Scots pine (*Pinus sylvestris* L.) seedlings by analyzing a
73 set of metabolites and enzymes involved in free radical scavenging and cell wall synthesis. The results
74 indicate that the combined analysis of the specific activities and isoform profile of peroxidases, superoxide
75 dismutases, and glutathione peroxidases coupled with the amount variation of phenolic compounds
76 enabled to discriminate between stressed and control seedlings. This approach represents a promising
77 platform for the assessment of temperature stress in forest trees and could also enhance selection and
78 breeding practices allowing for plants more tolerant/resistant to abiotic stress.
79

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80 **Key words**: Abiotic stress, Enzyme activity, Forestry, Peroxidase, Temperature stress

81 ## 1. Introduction

82 Recent studies of global climate change suggest that there will be more intense, more frequent, and longer
83 lasting heat in upcoming climate. For example, coupled models of climate and terrestrial biosphere
84 functions predict a continuous increase in average global temperatures of between 1.5 and 4°C/100 years,
85 with CO_2 concentrations rising to between 800 and 1000 ppm. (Gunderson et al., 2000; Friedlingstein,
86 2006; Heyder et al. 2011). Environmental stress factors such as drought and over-irradiation due to natural
87 conditions as well as to agronomic practices such as selective pruning or forest thinning that influence
88 albedo and temperature of the uncovered soils can be expected to become increasingly critical for forest
89 trees growing in central Europe. Changes in temperature are among the most important factors that affect
90 early plant development and their health. Plants have evolved mechanisms to monitor their environment
91 and to respond at cellular, physiological, and morphological level to optimize growth and reproductive
92 success within their lifespan (Taiz and Zeiger. 2010). Measurements of variations in enzymatic activity,
93 substrate specificity, and tissue localization of key enzymes represent valuable approaches to assess the
94 intensity of applied stress. . Most of the literature concerning stress factors in plants describes data
95 collected using model plants, crops and, rarely, fruit and industrial trees such as poplar (Bray. 2004,
96 Cohen et.al. 2010, Fortunati et.al. 2008, Qureshi et.al. 2007). Only recently the quantitative variation of
97 proteins in European beech grown under ozone stress has been described (Kerner et.al. 2014). The
98 available surveys indicate that three main groups of proteins might be particularly predictive, namely
99 enzymes involved in stress and defense metabolisms, such as PR-2 and PR-10 proteins, as well as storage
100 proteins. Specifically, the plant antioxidant response seems to be rapidly activated by heat (Ferreira et.al.
101 2006) and cold (Dai et.al. 2009) conditions. In adult beech trees (Haberer et.al. 2007) and in young beech
102 plants (Herbinger et.al. 2007) antioxidative compound variations were studied in a free-air controlled
103 ozone exposure system. However, the identification of reliable protein biomarkers suitable for evaluating
104 quantitatively the actual impact of different environmental stress conditions in forest plants is still to be
105 achieved. Glutathione and the enzymes involved in its turnover have been considered as potential stress

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106 biochemical makers of forest tree species because of their role in ozone detoxification (Dizengrem el et.al.
107 2013) but no experimental evidence has been brought to support this diagnostic approach. Nevertheless,
108 DNA expression and proteomic data (Gonzalez-Martinez et.al. 2007, Quareshi et al. 2007) suggest to
109 focus the research towards reactive oxygen species (hereinafter: ROS) and the enzymes involved in their
110 metabolism. In this perspective, peroxidase activity might be critical because it is involved in the active
111 oxygen consumption during cell wall anabolisms. Furthermore, metabolomic indicators such as
112 chlorophylls, carotenoids, tocopherol, ascorbate and glutathione (Tausz et.al. 2001, Tausz et.al. 2007) and
113 specifically phenol content could represent a supplementary class of valuable biomarkers since their level
114 and composition is affected by abiotic stress (Jansen et.al. 2014, Ramakrishna and Ravishankar. 2011)
115 The European beech (*Fagus sylvatica* L.) is one of the most important broadleaved deciduous trees in
116 central Europe, covering about 12 million ha, and providing a number of ecosystem services. It is widely
117 distributed from Scandinavia to Southern Italy and from Spain and Southwestern France to Ukraine
118 (Teissier du Cros et.al. 1981). Distribution of beech trees is likely to change due to modifications in
119 climate and water availability (Stojanović et.al. 2013) The Scots pine (*Pinus sylvestris* L.) is one of the
120 most important and widely distributed coniferous species (Bennett. 1984) Vidakovic. 1991).Changes in
121 quantity and quality of beech and pine wood could have a substantial ecological and economic impacts.
122 Consequently, the identification of both qualitative and quantitative stress-related biomarkers suitable for
123 validating different stress models adapted to these species is of outmost importance for forest industry and
124 farming. Apart from industrial importance, these trees are highly abundant key-species in temperate
125 central (beech) and boreal European forest ecosystems and therefore represent crucial indicators for better
126 assessing the overall forest health and ecosystem functioning.
127 The aim of this study was to identify appropriate biochemical markers able to report the "heat stress"
128 condition within beech and pine seedlings. This was achieved by comparing several biochemical
129 parameters in the tissues of seedlings grown at conditions that should reproduce the microclimate of both
130 natural environment and heat stresses. The biomarkers we looked for were characterized by reliability and

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131 simplicity in terms of manipulation, output, and data analysis since they were selected for clear
132 discrimination between stress classes rather than for their role in plant (stress) physiology.
133
134 **2. Material and methods**
135
136 **2.1. Plant material**
137 Pine (provenance Velika Gmajna 4.0253; 240-440 m a.s.l., 46°17' N 14°22' E) and beech (provenance
138 Osankarica 2.0119; 1240 m a.s.l., 46°27' N, 15°23' E) were planted on 20.11.2009
139 and grew outdoors for one year (tree nursery Omorika d.o.o., Muta, N Slovenia) before being transferred
140 to the Forestry Institute in Ljubljana, central Slovenia. Initially, 17 seedlings were selected for each
141 different condition (85 in total). Pines were planted in pots, and beech seedlings were planted as one
142 seedling per rhizotron. Rhizotrons (external size 30x50x3 cm) were packed in boxes and protected from
143 the light. The bottom third of the rhizotrons was filled with sand to allow for water draining, while the
144 upper two thirds were filled with dystric cambisol originated from sandstone and slate ground rock
145 collected from the upper soil horizon (0 to 30 cm) in a mixed forest in the vicinity of the Slovenian
146 Forestry Institute. The soil was sieved through a 5x5 mm sieve, autoclaved and mixed with one third of
147 vermiculite. No fertilizer was used during the experiment. After transplanting, seedling survival was 98%
148 and 95% in the successive growing season. Soil temperature was monitored at a depth of -20 cm using
149 factory calibrated digital temperature sensors DS18B20 connected to a datalogger developed at the
150 Slovenian Forestry Institute. Air temperature and humidity were logged by USB dataloggers Voltcraft®
151 DL-120 TH (Conrad Electronic UK Ltd, Barking, UK). CO_2 concentrations were monitored daily between
152 11.00-14.00 during the whole growing season 2011 using infrared gas analyzer Li-840 (Li-Cor Inc.,
153 Lincoln, USA). Seedlings were collected on 22.8.2012 experimentally measured growth conditions are
154 summarized in Table 1.
155 Pine seedlings were grown in three different conditions. Control samples (hereinafter CN, 10 samples
156 used for biochemical analyses) were grown outdoors sheltered from direct wind, whereas the

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157 experimental conditions presented either growth in a climatized room (IMP Klima, Godovič, Slovenia) at
158 air temperature of 16-20°C during the day (hereinafter CR, samples used for biochemical analyses) or
159 kept in the greenhouse where mean temperature was 12.3°C in December and Januar and fluctuated
160 between 40 and47°C in summer months (heat-stress, hereinafter HS, 8 samples used for biochemical
161 analyses) (Table 1).
162 Beech samples were grown under four different temperature conditions, three were the same as for the
163 pine samples (in all the cases, 7 samples were used for biochemical analyses) while the fourth group was
164 grown in a climatized room (IMP Klima, Godovič, Slovenia) at 16°C, with roots additionally cooled to
165 approximately 12°C (6 samples, hereinafter CR, all 6 samples used for biochemical analyses) by means of
166 a customized refrigerator. The treatment with additional cooling was used to mimic the natural
167 temperature gradient from aboveground to belowground. In the treatment without cooling of the root
168 system, the temperature of the root was practically equal to the temperature of the air. Samples in
169 climatized room were exposed to artificial light of a full PAR spectrum in the range 90 ± 5 µmol $m^{-2}s^{-1}$.
170 Seedlings grown in the green house were exposed to the natural light in the range 50 ± 3 µmol $m^{-2}s^{-1}$,
171 while seedlings grown outdoor were exposed to the natural light in the range 120 ± 20 µmol $m^{-2}s^{-1}$. The
172 daylight regime in the climatized room was adjusted weekly to the natural daylight regime. Beech
173 seedlings were watered with filtered tap water without additional nutrients via an automatic watering
174 system that kept the soil moisture at 10-15% during the growing season, while pine seedlings were
175 watered by hand daily so that the substrates were kept moist according to the consumption. After two
176 growing seasons, leaves and needles were randomly collected, snap frozen in liquid nitrogen, and stored at
177 -80ºC until further analysis. Prior to snap-freezing, weight and surface area of fresh material was
178 determined. In total, 53 samples of leaves/needles were measured..
179

180 **2.2. Homogenization and extraction of plant material**

181 Working temperature throughout the experiment was 4ºC. Homogenization was performed on ice using
182 mortar and pestle. Solvents used for homogenization were precooled overnight at 4°C (Bollag, 1996).

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183 Prior to freezing, each individual leaf/needle was weighed and area/length measured. Individual leaves or
184 needles were homogenized using 1mL of appropriate solvent per 0.1 g of weighed material (Table 2).
185 Frozen material was thawed during homogenization procedure in cooled extraction buffer. For analysis of
186 enzymatic activity and protein content plant material was extracted with extraction buffer (50 mM Tris-
187 HCl buffer, pH 7.4), while 80% methanol in water was used for the analysis of phenolics and free radical
188 scavenging activity. Plant homogenate was mixed at 4°C for 2 hours after which it was centrifuged 10 min
189 at 14,000x*g* at 4°C. Supernatant was used for the experiments after determination of total protein
190 concentration by using Quant-IT^{TM} protein assay (Life Technologies, USA) according to the
191 manufacturer's instructions.

192

193 **2.3. Determination of total phenolic content**

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

200

201 **2.4. Free Radical Scavenging Activity (FRSA) test**

202 The ability of plant methanol extract to scavenge free radicals was determined using di(phenyl)-(2,4,6-
203 trinitrophenyl) iminoazanium (DPPH) assay. The DPPH assay was performed according to the method
204 developed by Brand-Williams (Brand-Williams et.al. 1995). A solution of 50 µM DPPH in 80% (v/v)
205 methanol was stirred for 40 min. Then, 0.05 mL of standard or sample was mixed with 0.1 mL of DPPH
206 solution and incubated for 30 min in the dark after which the absorbance at 492 nm was detected using a
207 Perkin Elmer HTS 7000 spectrophotometric reader. The concentration of DPPH in the reaction medium
208 was calculated from a calibration curve using galic acid as a reference (concentration of 10 to 100 µg/mL

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209 in an 80% methanol solution, tested under the same conditions). The total free radical scavenging activity
210 was expressed as µg galic acid equivalents per mL of sample. All measurements were carried out in
211 triplicate.
212

213 **2.5. Measurement of enzyme activities**

214 Peroxidase activity (POX) was detected using o-dianisidine (Sigma-Aldrich, Germany) as a substrate
215 (Pine, Hoffman. 1984). The reaction mixture was prepared by mixing 20 mL of 50 mM phosphate buffer,
216 pH 7.0 with 9.79 mM hydrogen-peroxide and 0.2 mL o-dianisidine solution in methanol 11.1 mM. The
217 reaction was initiated by adding plant extract (50 µL) to the reaction mixture (900 µL) in the measuring
218 cuvette. The mixture was mixed and the change in absorbance at 430 nm was read for 5 min at RT using a
219 Perkin Elmer lambda 35 UV/Vis spectrophotometer (Perkin Elmer, USA). One unit of peroxidase activity
220 was defined as the amount of the enzyme that oxidizes o-dianisidine into 1 µM of bis-(3,3´-dimethoxy-4-
221 amino) azodiphenyl per min at 25ºC with the extinction coefficient 30 mM^{-1} cm^{-1}. Triplicate
222 measurements were performed for each sample.
223

224 Superoxide dismutase (SOD) activity was assayed by its ability to inhibit photochemical reduction of
225 nitrobluetetrazolium (NBT, Serva, Germany) to blue formazan (Winterbourn et.al. 1975). The reaction
226 mixture contained 50 mM phosphate buffer, pH 7.8, 0.66 mM EDTA, 10 mM $_{L}$-methionine, 33 µM NBT,
227 and 3.3 µM riboflavin. The reaction was initiated by adding plant extract (50 µL) to the reaction mixture
228 (200 µL). After mixing, samples were illuminated with sunlight for 10 min and absorbance at 492 nm was
229 recorded using HTS7000 Bioassay reader (Perkin Elemer, USA). Blank was prepared by mixing
230 extraction buffer with reaction mixture and kept in the dark while positive control was prepared in the
231 identical manner and exposed to sun light same as the samples. One unit was defined as the amount of
232 protein causing a 50% inhibition of NBT photoreduction. Triplicate measurements were performed for
233 each sample.
234

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

243

244 **2.6. Native PAGE and SDS PAGE**

245 For in-gel analysis of enzymatic activity, aqueous plant extracts were resolved under non-reducing
246 conditions in a discontinuous buffer system using a vertical electrophoresis slab system (Hoefer,
247 Holliston, USA) with a 4 % (w/v) stacking and a 10 % (w/v) resolving gel (Table 2). Each gel lane was
248 loaded with 12.5 μg of total protein. POX activity was identified according to a modified Quesada
249 protocol (Quesadaet.al. 1990). After electrophoresis, the gel was washed twice with 50 mM acetate, pH
250 6.0, after which it was incubated 1 h in 50 mM acetate, pH 6.0, 28 mM o-dianisidine, and 36.4 mM H_2O_2.
251 SOD activity after native PAGE was detected as previously described (Beauchamp and Fridovich. 1971)
252 For the analysis of protein profiles, plant samples were resolved using reducing SDS-PAGE and the same
253 gel conditions and protein amounts described above. Gels were stained with Coomassie Blue (Serva,
254 Heidelberg, Germany).

255

256 **2.7. Statistical analyses**

257 Statistical analysis was performed using GraphPad Prism v5.03 for Windows (San Diego, California,
258 USA). A significance level of $p \leq 0.05$ was used for analysis of variance, implemented using the Kruskal-
259 Wallis test followed by the Tukey's post-hoc test ($p \leq 0.05$). Correlation between different parameters
260 was performed at significance level of $p \leq 0.05$. Comparable percentage was calculated by the formula 3

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261 (%)= (100* sample/ control)-100 with positive values in the case of increase and negative values in the
262 case of decrease in comparison to the control. Unsupervised hierarchical clustering and heatmap
263 generation was accomplished using XLSTAT, with the Manhattan method and Pearson correlation for the
264 distance measure. For heatmap generation, measured values were standardized across the two cohorts by
265 conversion to Z-scores (peak height-mean/standard deviation).
266

267 3. Results

268

269 3.1. Morphological and biomarker changes in beach samples during temperature stress

270 Each individual leaf/needle (fresh plant material) was weighed and its area/length measured prior to
271 freezing (Fig. 1, Fig 5.). Significant differences in fresh weight (FW) were observed only in the cases of
272 beech CR . Specifically, beech CR seedlings produced heavier leaves (comparable percentage change
273 +93,05%, Fig 1A) No significant deviation from the control sample group was observed when leaf area
274 was measured (Fig. 1B).
275 A significant increase in phenolic content relatively to the control was observed for both CR and HS
276 samples (CR +43.81%, HS +37.33%), while CS samples did not show any significant change (Fig. 1C,
277 Fig. 5). A clear difference is observed in the beach plant extract ability to scavenge free radicals. In CS
278 plants it dropped significantly (-6.70%) while the free radical scavenging activity largely increased in CR
279 (+101.65) and HS (+97.93%) samples in relation to control samples (Fig 1D, Fig. 5).
280 The POX specific activity was clearly affected by artificial growth conditions (Fig. 2A, Fig. 5A). A
281 significant decrease in POX specific activity was observed when comparing the samples grown outside
282 (CN) with samples grown inside the climatized room both with and without root cooling (CS and CR, by -
283 51.71% and -59.62%, respectively). The statistical significance of the POX activity variation further
284 increased in samples treated to reproduce a heating stress (HS, by -70.49%). SOD specific activity
285 decreased significantly in the samples subjected to all of the applied conditions (Fig. 2B, Fig. 5A) when

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286 they were compared to control plants (CS -46.16%, CN -62.47%, HS -62.96%) . GPX activity increased
287 only in beech CS samples (+97.52%) (Fig. 2C, Fig. 5A)..
288 Since plants possess several POX isoforms, we attempted to separate them using a native gel system to
289 possibly identify which of the enzyme isoforms might be an appropriate biomarker specific for the
290 different growth temperature. A clear difference in the POX expression pattern was observed for beach
291 samples (Fig. 2D, POX). In The majority of POX activity is performed by isoforms with apparent high
292 molecular weight, whereas the contribution of low molecular weight isoforms seems to be negligible. In
293 the CS samples, a slight increase in the activity of low molecular weight isoforms was observed with no
294 apparent decrease in activity of the high molecular weight isoforms. In contrast, the CR seedlings showed
295 both the loss of some among the high molecular weight isoforms and a relevant enhancement of the low
296 molecular weight isoform activity. A qualitatively different POX isoform profile was identified also in the
297 samples extracted from seedlings subjected to heat stress (HS).
298 Native PAGE was used also to separate SOD isoforms (Fig. 2D, SOD). However, for all of the samples no
299 exploitable difference in isoform profile was observed and the measured differences in specific activity
300 seems rather to be attributed to the different level of expression of the same SOD isoform.
301 Such information could be integrated by the analysis of the sample total protein profile determined by
302 SDS PAGE that indicated distinctive differences in all of the analyzed beach samples (Fig. 2D, SDS).
303

304 **3.2 Morphological and biomarker changes in pine samples during temperature stress**

305 Changes in pine needle size was observed only in case of CS plants (change of -59,38%) (Figs. 3A and
306 5B) while HS samples were unaffected. Similar results were observed for needle weight. CS plants
307 showed decreased needle weight (-59,38%, Fig 3B and 5B) while HS again showed no significant change.
308 In the case of phenolic content, no statistically significant difference between the samples and the control
309 was observed in pine samples (Fig. 3B, Fig. 5B). A highly significant increase in free radical scavenging
310 activity was noted only for the CS seedlings (+65.66%) (Fig 3B, Fig. 5B).

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311 POX activity in pine seedlings was unaffected by heat stress response (Fig. 4A, Fig. 5B), whereas a
312 dramatic decrease was recorded for seedlings which grew in strictly controlled conditions (change of -
313 96.67%) throughout their growing season. The most significant decrease in SOD specific activity was
314 observed in seedlings subjected to heat stress (Figs 4B and 5B, -54,60%). Only heat stress conditions (HS)
315 induced a decrease in GPX activity (Fig 4C-29.86%) while such enzymatic activity remained unaffected
316 in seedlings that underwent growth conditions in which air temperature was controlled (Figs 4C and 5B;
317 CS).
318 Native PAGE was used for evaluation of POX specific activity of pine seedling extract (Fig 4D, POX).
319 There is an observable difference among the tested samples that seems to be due to the relative
320 expression/activity level of the isoforms rather than to the appearance/disappearance of specific isoforms.
321 Nevertheless, the overall patterns appear very specific for each of the growth conditions. Same as in the
322 case of beech samples, analysis of total protein profile via SDS-PAGE shows a distinct profile (Fig 4D,
323 SDS) for each of the analyzed samples.
324
325 **4. Discussion**
326 In this study we evaluated the possibility of using simple biochemical techniques to recover reliable data
327 able to differentiate control seedling samples from plants subjected to different temperature stresses and
328 applied this analysis to two industrially important and highly abundant tree species, the European beech
329 and the Scots pine. In this perspective, the choice of the biomarkers can be totally arbitrary and their
330 physiological relevance could be also minimal. What is really crucial is their capacity to act as specific
331 indicators of a stress condition by clearly discriminating between stressed and control samples.
332 Furthermore, biomarkers should be robust in the sense that their recovery, processing, and analysis should
333 be simple and allow for large-scale data comparison among samples collected under very variable field
334 conditions. The evaluation of POX activity and its isoforms was of logical choice and we thought to
335 integrate it with the analysis of other molecules involved in ROS scavenging and cell-wall metabolism.

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336 The statistical analysis indicates that both enzyme biomarkers such as peroxidases, superoxide dismutases,
337 and glutathione peroxidases and phenolic compounds were able to discriminate between stressed and
338 control seedlings. Dendrogram and accompanying heatmap further depict the relative incicence of
339 biomarkers in stressed and control seedlings (Fig. 5). There is a very high correlation between SOD and
340 POX specific activity in beech samples (r^2=0.9889, p= 0.0055). A strong positive correlation in beech is
341 also observed for phenolic content and leaf weight (r^2= 0.9758, p= 0.0121) as well as phenolic content and
342 FRSA(r^2=0.9157, p= 0.0421), and FRSA and leaf weight (r^2=0.9430, p= 0.0284). This is further
343 corroborated by clear clustering of these parameters in the heatmap (Fig. 5A). In pine samples strong
344 positive correlation is observed between GPX and SOD activity (r^2=0.9988, p= 0.0152), GPX and
345 phenolic concentration (r^2=0.9950, p= 0.0316), SOD and phenolic concentration (r^2=0.9886, p= 0.0163),
346 and between leaf size and weight (r^2=0.9923, p= 0.0393). This is also clearly visible in dendrogram and
347 accompanying heatmap (Fig. 5B).

348 Plant responses to abiotic stresses comprises morphological, physiological, and biochemical changes that
349 contribute to counteract the damages and facilitate the recovery of the impaired systems (Potters et.al.
350 2007, Stojnic S.et.al. 2015.). Recent studies revealed that plants are able to develop distinct morphological
351 responses (stress-induced morphogenesis responses – SIMRs) when challenged with abiotic stresses
352 (Potters et.al. 2007, Potters et.al. 2009). Different stressors were found to induce similar morphogenic
353 responses such as inhibition of cell elongation, localized stimulation of cell division, and modification of
354 cell differentiation (Potters et.al.2007). In other cases, increased temperature and drought did not affect
355 significantly plant biomass and did not cause variations of the weight and surface of the leaves/needles
356 recovered from the treated seedlings in Douglas-fir seedlings (Jansen et. al. 2014). On the other hand, soil
357 temperature influences beech root length, root tip density, biomass of shots and roots (Štraus et.al. 2014),
358 while stomal density (Stojnic. et. al. 2015.) seems to be strongly influenced (Štraus et.al. 2014) by water
359 availability. In contrast, the enzymes involved in the response to oxidative stress as well as the phenolic
360 compounds (Jansen et al. 2014) seem to be more suitable to be exploited as biomarkers to monitor
361 temperature-induced physiological modifications. Both controlled air temperature conditions and over-

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362 heating resulted in the very strong inhibition of POX and SOD total activities in beech samples. At least in
363 the case of POX, also the isoform expression and their activity profile could be easily used to gain
364 information concerning the level of stress within seedlings. The other tested enzymatic activities and
365 compound concentrations seem to be less suitable stress sensors in the sense that significant variations
366 have been measured only in particular cases, such as the increase of GPX activity in CS and the higher
367 phenol content and free radical scavenging activity specific of CR and HS beech plants. The measured
368 variations are confirmed by previous reports which indicated that plants most probably accumulate
369 secondary metabolite and enhance antioxidant capacity during acclimation (Cansev et.al. 2012) and in
370 response to higher temperatures (Rivero et.al. 2001).
371 The identification of reliable biomarkers for pine stress was less straightforward. Nevertheless, POX
372 activity strongly declined in the presence of CS conditions, whereas SOD – and to a smaller extent GPX -
373 activity dropped in the presence of heat stress. Native gels stained for POX activity were useful to
374 discriminate between control and stressed samples since the intensity of the different isoforms was
375 strongly affected.
376 Total phenolic content was not significantly altered by growth conditions while FRSA test could be
377 considered as a biomarker in the case of stress induced by cold growth conditions.
378 In this work we aimed at identifying robust and reliable biomarkers to monitor stress conditions induced
379 by environmental factors. The elucidation of the physiological modifications that happen in beech and
380 pine under the stress conditions induced in our experiments is beyond the aim of the present contribution.
381 For instance, enzymes such as POX play both a scavenging activity and an anabolic role in the lignin
382 biosynthesis using hydrogen peroxide as a substrate (De Marco and Roubelakis-Angelakis. 1996) and
383 consequently the separate analysis of the activity and cellular localization of the different isoforms would
384 be necessary to understand their biological meaning . Used as a biomarker, POX enables to obtain very
385 clear and reproducible quantitative (specific activity) and qualitative (isoform profile) data to discriminate
386 samples by means of simple technical analyses. Quantitative measurements are useful to obtain data for
387 modeling whereas the isoform profile separated by native gels can be very informative to identify

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388 biomarkers specific for different stress factors . Furthermore, our results indicate that coupling "POX
389 fingerprinting" with data relative to one or few other parameters, such as SOD activity, phenolic content
390 and FRSA for beech, and GPX activity and phenolic content in pine, could define particular combinations
391 of stress conditions. The present report might act as a sort of proof-of-principle for the future
392 accomplishment of larger sets of biochemical biomarkers for different species of forest tree species.
393 Assessing the health of individual plant specimens on molecular/metabolic level could then give overall
394 indication of environmental conditions in a forest and allow for the improvement of the methods used for
395 forest health monitoring and management (Trudić et.al. 2012). Given the test simplicity, it would be
396 worthy to apply POX analysis to other relevant stress conditions for forest plants such as the presence of
397 pollutants in the air or in the soil.
398
399 ## 5. Conclusions
400 The collected results indicate the feasibility of identifying biochemical biomarkers to use for evaluating
401 the effect of temperature modification on the seedlings of forest tree physiology. Identified biochemical
402 biomarkers could be integrated with genetic information (Fang et.al. 2014, Liu et.al. 2014) to provide
403 useful data needed for assessing environmental stress and for modeling the ecological impacts of global
404 climate change on European beech and Scots pine across their distribution areas. Furthermore, coupling
405 the variation of peroxidase isoform profile with other enzymatic parameters could represent a simple,
406 reproducible, and possibly more universal assessment method for monitoring the tolerance level to
407 temperature stress (in other forest plant seedlings. This approach developed for seedlings might represent
408 also a promising platform for the evaluation of heat stress in adult forest plants in combination with
409 orthogonal techniques such as genomic and proteomic analyses.
410 These tools could also improve selection and breeding practices since they simplify the profiling of clones
411 to identify those that are more tolerant/resistant to abiotic stress.
412
413

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420 growth

421

422 **Compliance with ethical standards**

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

427

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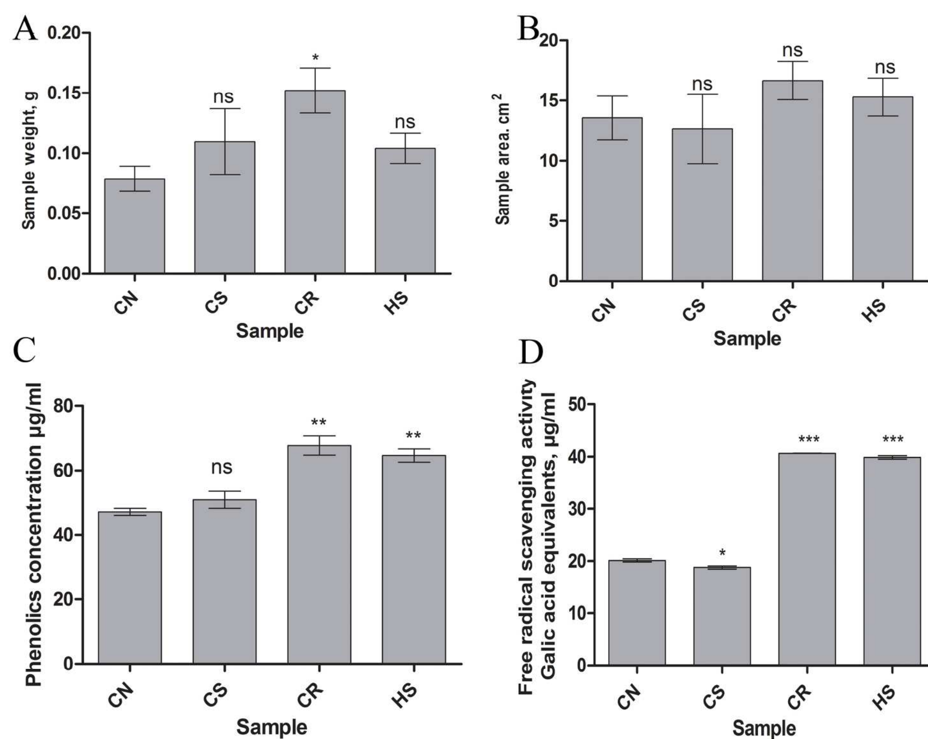

Figure 1. Comparison of morphological characteristics and determination of total phenolic content and free radical scavenging activity of plant methanol extracts for beech seedlings
Plant fresh weight is expressed in grams (A); Plant surface is expressed in cm^2 (B). Total phenolic concentration in plant methanol extracts is expressed in mg/ml (C); Free radical scavenging activity in plant methanol extract expressed as galic acid equivalents in mg/ml (D). The errors bars indicate standard deviations for triplicate measurements

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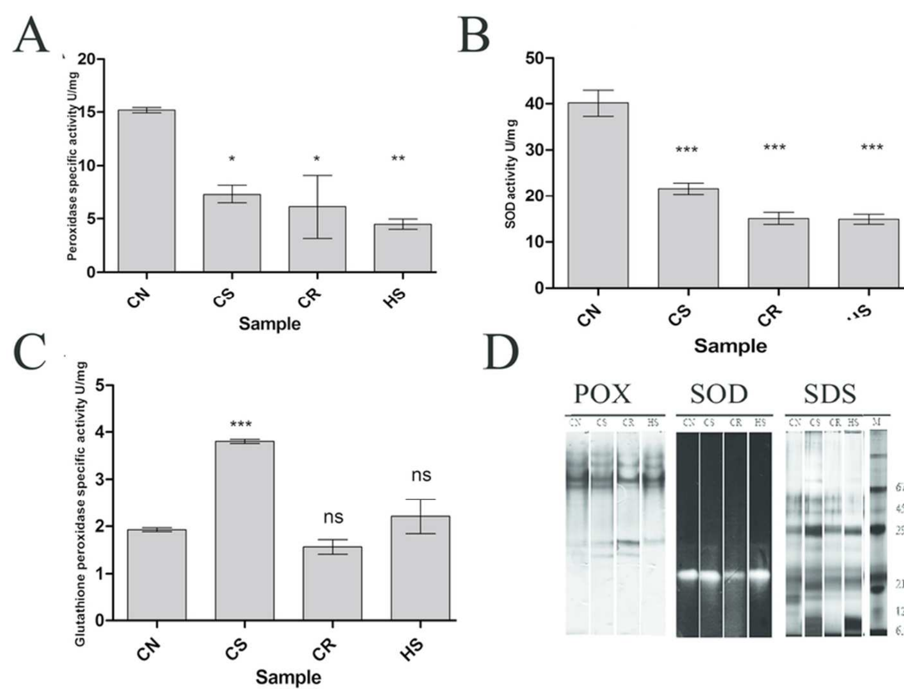

Figure 2. Specific activity of biomarker enzymes in soluble protein extract and electrophoretic separation of biomarker isoforms and total protein content in beech seedlings
Specific enzymatic activities of peroxidase (A), superoxide dismutase (B), glutathione peroxidase (C) are represented in the graph. The errors bars indicate standard deviations for triplicate measurements. Detection of peroxidase (D; POX) and superoxide dismutase (D; SOD) by native PAGE zymography, SDS PAGE (D; SDS) of total soluble extract.

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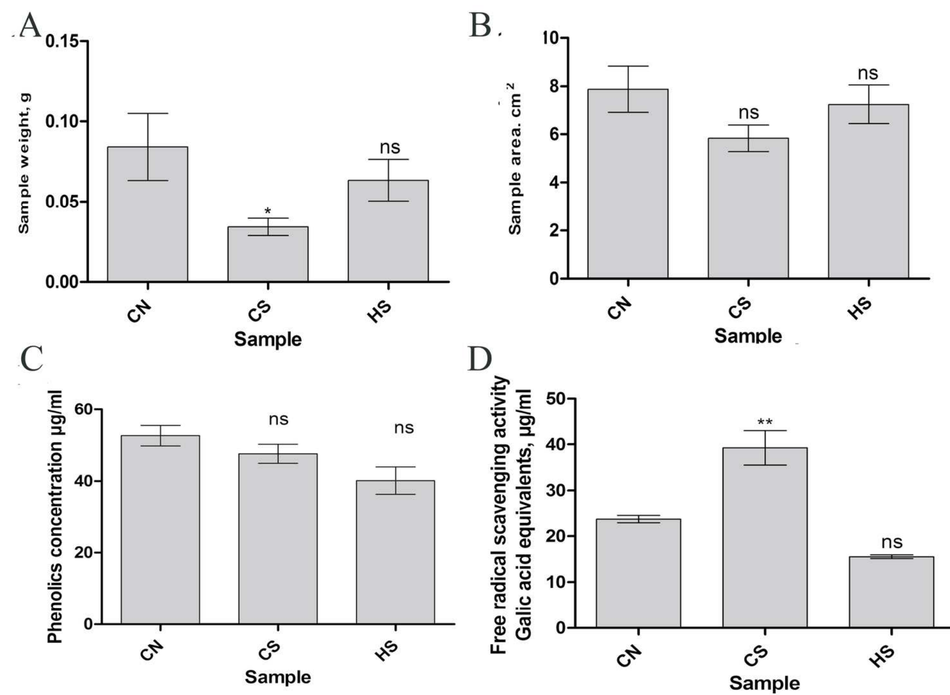

Figure 3. Comparison of morphological characteristics and determination of total phenolic content and free radical scavenging activity of plant methanol extracts for pine seedlings
Plant fresh weight is expressed in grams (A); Plant surface is expressed in cm^2 (B). Total phenolic concentration in plant methanol extracts is expressed in mg/ml (C); Free radical scavenging activity in plant methanol extract expressed as galic acid equivalents in mg/ml (D). The errors bars indicate standard deviations for triplicate measurements

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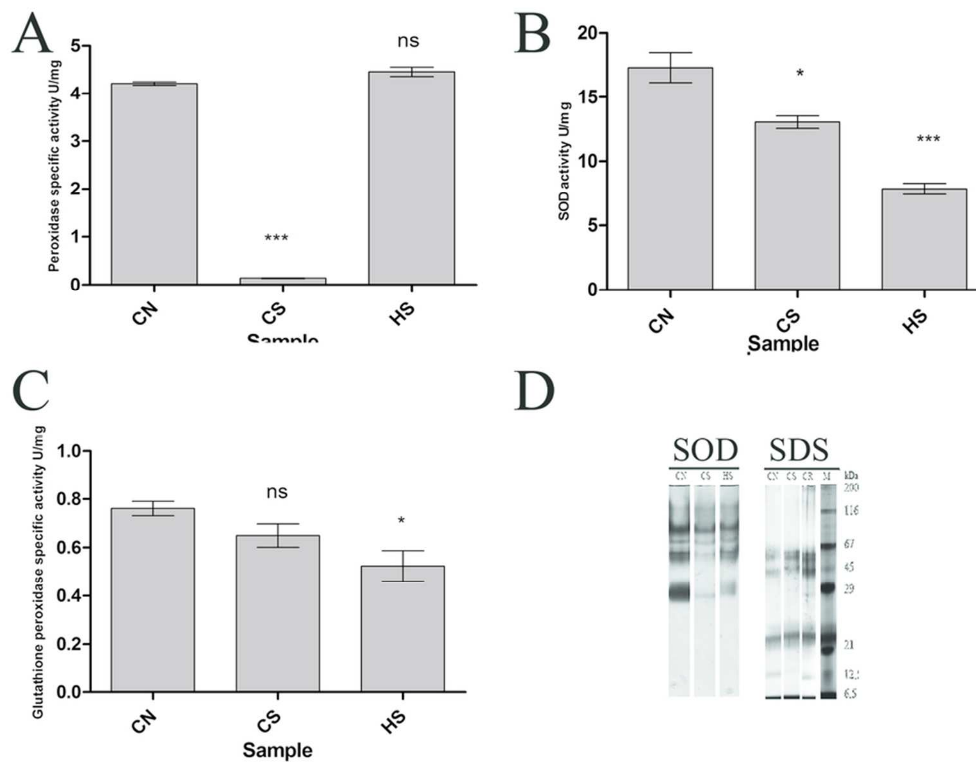

Figure 4. Specific activity of biomarker enzymes in soluble protein extract and electrophoretic separation of biomarker isoforms and total protein content in pine seedlings
Specific enzymatic activities of peroxidase (A), superoxide dismutase (B), glutathione peroxidase (C) are represented in the graph. The errors bars indicate standard deviations for triplicate measurements. Detection of peroxidase (D; POX) and superoxide dismutase (D; SOD) by native PAGE zymography, SDS PAGE (D; SDS) of total soluble extract.

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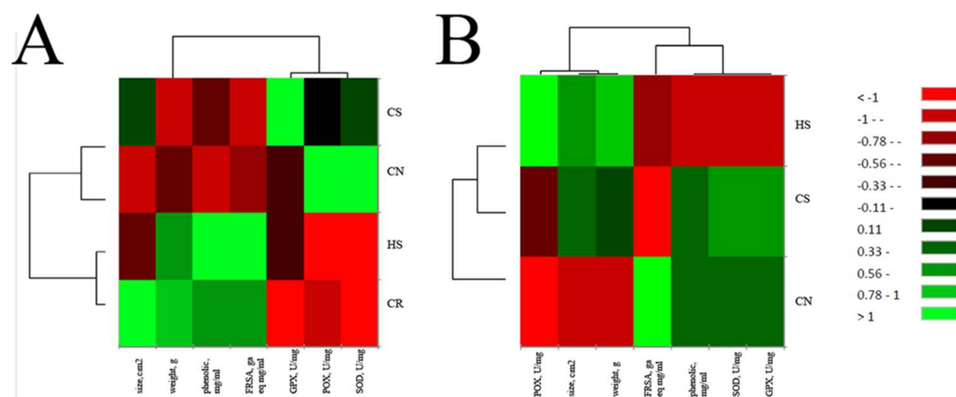

Figure 5. Heat map showing unsupervised hierarchical clustering of different biomarker values according to the seedling growth conditions
Measured biomarkers for beech (A) and pine (B) seedlings are arranged in colums while growth coditions are in rows. Shades of red represent elevation of a metabolite while shades of green represent decrease of a bimarker value, relative to the median levels (see color scale). In the dendrograms, the clustering clearly differentiates the stressed and control samples. Plant fresh weight is expressed in grams (weight, g); Plant surface is expressed in cm^2 (size, cm^2). Total phenolic concentration in plant methanol extracts is expressed in mg/ml (phenolics, mg/ml); Free radical scavenging activity in plant methanol extract expressed as galic acid equivalents in mg/ml (FRSA 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

CN: Samples grown outdoor, CS: Samples grown at 16°C, CR: Samples grown at 16°C with roots cooled to 12°C, , HS: Samples grown in green-house

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)		
		Mean ± SD	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**	**Mean ± SD**	**Min.**	**Max.**
Climatized room	**CS**	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
	CR										12.0 ± 2.9	9.1	25.9
Greenhouse	**HS**	20.1 ± 7.4	10.9	47.1	56.1 ± 14.0	10.3	86.1	532 ± 80	415	792	21.5 ± 5.0	8.6	31.6
Outdoor	**CN**	10.2 ± 9.0	-12.0	29.6	79.4 ± 15.4	17.8	99.7	413 ± 25	364	482	15.2 ± 6.8	-0.2	26.6

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15 Table 2: Experimental set-up

16 POX: peroxidase, SOD: superoxide dismutase, GPX: glutathione peroxidase, FRSA: free radical scavenging activity

Tree Species	**European beech (*Fagus sylvatica* L.)**		**Scots pine (*Pinus sylvestris* L.)**	
Morphological features	Leaf size; Leaf weight			
Extraction procedure	50 mM Tris-HCl buffer, pH 7.4	80% methanol	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, SOD Total soluble protein profile (SDS-PAGE)	Phenolic concentration (µg/mL) DPPH RSA test	Enzyme specific activity (U/mg): POX, SOD, GPX Enzyme isoform distribution (Native PAGE): POX, SOD Total soluble protein profile (SDS-PAGE)	Phenolic concentration (µg/mL) FRSA test