Additional Information

Biochemical responses to drought, at the seedling stage, of several Romanian Carpathian populations of Norway spruce (*Picea abies* L. Karst)

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Abstract. Norway spruce is a native European coniferous species distributed from the Carpathian Mountains and the Alps to northern Scandinavia. In the coming decades, spruce forests will need to cope with increasing climate changes which are already threatening their natural habitats. European forest policy should adapt to the changing climate by deploying drought-tolerant genotypes in regions expected to experience increased stress in the future. To identify reliable water stress biomarkers in this species, which may be eventually used to select populations responding better to forecasted drought events, we studied the physiological responses to severe water stress treatments of spruce seedlings originating from several locations in the Romanian Carpathian Mountains. One-year old seedlings were denied irrigation for six weeks in order to generate severe water stress in controlled conditions. Variations in the levels of the studied photosynthetic pigments, osmolytes, and non-enzymatic antioxidants were detected across the spruce populations. Several of the determined parameters in seedling needles, such as the decrease in water content (nearly 40% decrement in the most sensitive studied populations), the degradation of chlorophylls, or a low increase of proline levels (up to seven-fold increment in the most sensitive populations in comparison to no change in the most tolerant ones), could be employed as biomarkers for an early assessment of water stress at this stage. Furthermore, seedlings from two of the populations under study (Sudrigiu and Gioristea-Calimanut) apparently responded better to water stress than the other populations, as shown by their lower reduction in needle water content, no degradation of chlorophyll *a* and carotenoids, and less proline accumulation, suggesting a relatively higher

resistance to drought. These populations also seemed to be the least affected by osmotic stress at the seed germination stage. Therefore, we consider that the use of biochemical markers of stress at early seedling stages could represent a useful tool for the initial screening of populations that have relatively high tolerance to drought, warranting further research for potential use in reforestation.

Key Message: Norway spruce seedlings apparently showing a relatively higher tolerance to drought can be easily selected using a battery of biomarkers such as water content, chlorophyll and proline levels in the needles, and could be eventually used as initial screening method in reforestation programmes.

55   **Authors Contributions:** The experiments were designed and conceived by STS, MAH, MB and

56   OV, and performed by STS and MAH. All authors contributed to the analysis of the results, the

57   statistical treatment of the data and the preparation of the manuscript. OV was responsible for the

58   general supervision of the work. The reagents and materials used to carry out the experimental work

59   were contributed by MB and OV.

60

61   Whereby,

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69   **Conflict of Interests:** The authors declare no conflict of interests.

1. Introduction

Norway spruce is a native European coniferous species growing in large areas across north Scandinavia, the Alps, and the Balkan and Carpathian Mountains (Mitchell 1972). It has an estimated geographical range of approximately 30 million hectares, while at least 7 million hectares of pure spruce are located outside its natural range (Jansson et al. 2013; Kazda 2005).

In the coming decades, the world's forest trees will need to cope with the increasing climate changes which are already threatening their natural habitats (Allen et al. 2010; Dale et al. 2001; Gilliam 2016). Consequently, new strategies and measures should be designed and implemented to minimise wood and environmental losses in the future. Europe's spruce reforestation policies should be based on the so-called 'adaptive forest management', a relatively recent concept regarding species survival and evolution (Lindner 2000). Active adaptation would be a suitable management strategy for stand conversion, based on replacing sensitive populations or tree species with others which could potentially respond better to the forecasted climate changes during their life cycle (Bolte et al. 2009).

The distribution of Norway spruce has varied in time due to climate influence (Bradshaw et al. 2000). The deleterious effects of environmental stresses, especially drought, on growth rates and tree mortality have often been underestimated in the past (Spiecker 2000). The occurrence of previous drought periods has damaged spruce stand in terms of radial growth, particularly in areas outside its natural range, which are affected by lower precipitation levels (Kahle et al. 2005).

The Intergovernmental Panel on Climate Change (IPCC) climate predictions up to 2080 show that mean temperatures will increase on the continent by 1.4-4.5 °C, with the largest differences occurring in the southern countries (EEA 2004). According to Cuculeanu et al. (2002), who analysed climate change in Romania until 2075 using different GCMs (General Circulation Models), air temperature in Romania will increase by 2.8-4.9 °C, depending on the model used, while precipitation will decrease by 20 %. As reported by Lévesque (2013), Norway spruce populations from Central Europe are more vulnerable to soil water deficit than other conifers. The species will likely migrate to higher altitudes and to the north of Europe, with great area losses in the central part of the continent (Hanewinkel et al. 2013). Other studies confirm that spruce evaporative requirements are strongly related to the water supply of the stand, while drought sensitivity seems to be higher in altitudes below 1000 m (Maaten-Theunissen et al. 2013).

Spruce stands across Europe, including those in Romania, are likely to be affected by drought stress in the near future, with negative effects on growth rate and timber quality. Additional damage to the forest could involve lower rates of natural regeneration and reduced natural productivity, and possibly the disruption of its ecological balance and composition. The risk of infestation by insects and fungal diseases, such as spruce bark beetle or *Armillaria* spp., may weaken stand stability, causing more wind breakage events (Green and Ray 2009; Hart et al. 2014; Kolström et al. 2011).

104   The most common drought effects include inhibition of photosynthesis due to reduction of

105   photosynthetic pigment contents, stomatal oscillations, respiration and oxidative damage (Farooq et al. 2009;

106   Jaleel et al. 2009). Genetic approaches have shown that drought tolerance of plants relies on their capability

107   of activating adaptive mechanisms to counteract these deleterious effects, at physiological, biochemical and

108   molecular levels (Kantar et al. 2011).

109   Spruce trees affected by drought show parallel biochemical and physiological changes leading to

110   reductions in sap flow, in the rate of stem circumference increase, and in needle water potential (Ditmarová

111   et al. 2010; Ježík et al. 2014). At the cytological level, it has been observed that xylem cells have smaller

112   lumen and thicker walls, while the number of underdeveloped sclerenchyma cells increases (Kivimäenpää et

113   al. 2003; Montwe et al. 2014). Protective mechanisms in the first days of seedling exposure to drought

114   consist of an adjustment to low osmotic potential correlated with a decline in transpiration and activation of

115   nitrogen metabolism (Grossnickle 2000; Modrzynski 2007). Long term drought leads to hydraulic failure and

116   carbon starvation, and eventually to tree mortality (McDowell et al. 2008).

117   As a response to the forecasted effects of climate change, the 'European Information System on

118   Forest Genetic Resources' has established 363 gene conservation units and 202 seed stands across 14

119   European countries (EUFGIS 2011). Romania ranks in the second position, with 84 seed units which are used

120   in the country's reforestation programmes. These seed stands should be suitable for the specific local

121   environmental conditions. However, large areas of the country's Norway spruce stands have been affected by

122   drought, and it is necessary to consider an adaptive management approach for spruce reforestation.

123   Reforestation programmes in Europe are using spruce seedlings with origin in the certified seed stand

124   mentioned above. Yet forestry practices should take into consideration alternative solutions to minimise stand

125   losses and environmental damage. A selection of European spruce populations with higher drought tolerance

126   would provide a better chance for future stand development in regions projected to experience greater climatic

127   stress.

128   The effects of abiotic stress, including drought, are generally assessed by measuring the degree of

129   inhibition of plant growth. Faced with a situation of stress, plants divert resources (energy, metabolic

130   precursors) from normal metabolism and biomass accumulation, to the activation of defence mechanisms,

131   which causes the arrest or drastic reduction of growth (Munns and Termaat 1986; Zhu 2001). However, in

132   species of slow growth, like spruce and most forest tress, very long treatments would be required to observe

133   significant stress-induced changes in growth parameters. The definition of specific, easy to quantify,

134   physiological and biochemical markers associated with drought, should allow an initial, rapid analysis of the

135   responses of the plants to the stress treatment. Appropriate biomarkers are likely to include those involved in

136   conserved responses to water stress: a decrease in leaf water content, degradation of photosynthetic pigments,

137   the accumulation of specific osmolytes, or the activation of antioxidant systems, changes that have been

138   associated to abiotic stress-induced inhibition of growth in many different woody species (e.g., Jiménez et al.

139   2013; Popović et al. 2016; Zrig et al. 2015), including conifers (Guo et al. 2010; Schiop et al. 2015;

140   Sudachkova et al. 2002).

The major aim of this work was to identify suitable biochemical markers associated with drought in *Picea abies* seedlings, which could be used for the rapid analysis of responses to controlled water stress treatments. Several Norway spruce populations, located at different altitudes along the Romanian Carpathian Mountains, were selected for these experiments. Seedlings, obtained by germination of seeds with an established origin, were grown in the greenhouse before being subjected to water stress treatments. In complementary experiments, seeds from the same stocks were independently germinated *in vitro* under conditions of osmotic stress – to mimic the drought treatment. By performing a comparative analysis of the responses of different spruce populations, the study also aimed at developing a tool based on combination of several biochemical markers that could be used for a preliminary e selection of the populations that better respond to water stress at early seedling stage.

2. Materials and Methods

Seedling origin, growth conditions and water stress treatments

Descendants from seven Romanian spruce populations, included in the National Catalogues of Forest Genetic Resources and Seed Reservations, were grown in a greenhouse located in Albac, Romania, during one complete vegetation season (the origins of the genetic resources and climatic data are indicated in Tab. 1). The seeds were sown during April under appropriate conditions of temperature and high humidity of air and soil. Prior to sowing, the seeds were subjected to a pre-germination treatment, keeping them in cold water for 48 hours, as described by Radu et al. (1994). The seedlings were grown on peat substrate collected from a site in the Vladeasa Mountains, Romania, near the place known as Padis (46.60415 N, 22.69618 E).

Tab. 1 Climatic data in the regions of origin of the seven studied *P. abies* populations, according to the National Meteorological Administration (NMA), Romania (Schiop et al. 2015)

Identification	Population	Geographic coordinates	Altitude (m.a.s.l.)	Mean annual Temperature (°C)	Mean annual Precipitation (mm)	Mean potential PET (mm)
GC	Gioristea-Calimanut	46°45' N / 25°20' E	840-1200	6.4	642.0	558.5
VM	Valea Mare	46° 28' N / 23°09' E	1200-1450	5.6	893.0	517.3
SD	Sudrigiu	46° 36' N / 22° 25' E	230	10.4	751.9	672.6
BM	Basca Mica	45°45' N / 26°20' E	1250-1500	1.9	800.7	428.6
AB	Albac	46°45' N / 22°97' E	770	7.8	837.6	594.0
PT	ParaulTurculet	47°44' N / 25°24' E	850-930	7.2	707.8	607.3

JP	Jepi	45°15' N / 24°45' E	1350-1650	0.9	1303.2	360.4	

One-year-old seedlings were transplanted into pots with peat substrate during vegetative phase (blossom buds) and were transported to the greenhouse of the Institute of Plant Molecular and Cellular Biology (IBMCP), Polytechnic University of Valencia, Spain. Daily mean temperature during the 42 day-long experiment was 20 ^{o}C, photoperiod 16 hours of light, humidity ranged between 60-70 % and CO_2 level was approximately 300 ppm. Control plants were watered twice a week using Hoagland's nutritive solution while drought treatment was performed by completely ceasing irrigation. After 42 days of treatment, water-stressed and control seedlings were harvested and several physiological and biochemical traits were determined in the needles.

Osmotic stress treatments at the seed germination stage

Seeds from the same seven stocks used to grow the spruce seedlings, were germinated *in vitro*, in the presence of increasing concentrations of polyethylene glycol 6000 (PEG-6000). Petri dishes (diameter: 9 cm) were prepared with a sterile cotton base below a double layer of sterile filter paper, and 40 mL of water (for the control seeds) or 40 mL of aqueous PEG-6000 solutions generating osmotic pressures of -0.13 MPa (17.58 g/L), -0.26 MPa (30.12 g/L), or -0.52 MPa (49.37 g/L) were added to each dish. These osmotic pressures are the same produced by 30, 60 and 120 mM NaCl solutions, respectively, and were calculated based on the Van't Hoff's equation (Ben-Gal et al. 2009).

Seeds were surface-sterilised in 15% (v/v) hydrogen peroxide for one hour, then thoroughly rinsed with sterile water, and dried at room temperature. Sterilised seeds were placed on the surface of the filter paper in the Petri dishes, 30 seeds per dish, and the plates were sealed with parafilm. Three replicated Petri dishes (90 seeds in total) were used per spruce population and per treatment. Over a period of 21 days of germination, the plates were checked regularly (twice per week) and the number of germinated seeds was registered. At the end of the treatment, the seeds were scanned and the radicle, hypocotyl, and cotyledon lengths were measured using ImageJ software (Rasband 1997-2012). 'Mean germination time' (MGT) was calculated according to the formula: MGT= $\Sigma Dn/\Sigma n$, where n represents the number of seeds germinated at day D, while D is the number of days from the beginning of germination (Ellis and Roberts 1981). Seedling vigour index (SVI) was calculated as: Germination percentage x [Mean root length (mm) + Mean hypocotyl length (mm)], as indicated by Abdul-Baki and Anderson (1973).

Water content percentage

200   Water content percentage (WC%) in needles was obtained by measuring their initial fresh weight

201   (FW), and their dry weight (DW) – after drying the needles in an oven for 4 days at 65°C until constant

202   weight – using the formula: WC% = $[(FW - DW)/FW]$ x 100.

203

204   *Osmolyte levels*

205

206   Free proline (Pro) in fresh needles was quantified according to the ninhydrin-acetic acid method of

207   Bates et al. (1973). Pro was extracted in 2 mL of 3% aqueous sulfosalicylic acid; 1 mL of the extract was

208   mixed with one volume of acid ninhydrin and one volume of glacial acetic acid, and incubated at 95 °C for 1

209   h. The sample was extracted with two volumes of toluene and absorbance of the organic phase was

210   determined at 520 nm, with toluene as a blank.

211   Total soluble sugars (TSS) in needles were measured according to the method of Dubois et al.

212   (1956): ground dry material was suspended in 80 % methanol and the sample was mixed in a shaker

213   overnight. Concentrated sulphuric acid and 5 % phenol was added and the absorbance of the solution at 490

214   nm was measured.

215

216   *Non-enzymatic anti-oxidants*

217

218   Total phenolic compounds (TPC) and 'total flavonoids' (TF) were measured in the same methanol

219   extracts used for TSS determination. TPC were quantified by measuring the absorbance at 765 nm after

220   reaction with the Folin-Ciocalteu reagent, according to Blainski et al. (2013), and expressed as equivalents of

221   gallic acid (mg eq GA g^{-1} DW), used to obtain the standard curve. TF were measured following the procedure

222   described by Zhishen et al. (1999), based on the nitration of aromatic rings bearing a catechol group and their

223   reaction with $AlCl_3$; this method detects antioxidant flavonoids but also other phenolics containing a catechol

224   group. After the reaction, the absorbance of the sample was measured at 510 nm, and the amount of

225   flavonoids was expressed in catechin equivalents (mg eq. Catec. g^{-1} DW).

226

227   *Photosynthetic pigments*

228

229   Photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) were measured

230   according to the method of Lichtenthaler and Welburn (1983). The extraction was carried out in 80 % cold

231   acetone and optical density was read at 663 nm, 646 nm and 470 nm. The values were expressed in µg/ml and

232   then transformed to µg/g DW using the following formulas:

233

234   Chlorophyll a (µg/ml) = 12.21(A_{663})- 2.81 (A_{646})

235   Chlorophyll b (µg/ml) = 20.13 (A_{646}) - 5.03 (A_{663})

236   Carotenoids (µg/ml) = (1000 A_{470} - 3.27 [chl a] – 104 [chl b])/227

237

238 *Statistical analysis*

239

240   Statistical analysis of the data was performed using Statgraphics Centurion XVI software (Statpoint

241 Technologies, Inc.; Warrenton, Virginia, USA). The assumption of normality was checked by Shapiro-Wilk

242 W test and the homogeneity of variance by the Levene test. One-way ANOVA was performed to compare the

243 statistical significance of the difference among treatments at confidence level of 95%. When the ANOVA null

244 hypothesis was rejected, post-hoc comparisons were performed using the t-test (for seedling experiments) and

245 the Tukey HSD test (for *in vitro* germination assays). All means throughout the text include the standard

246 deviation (SD)

247

248 **3. Results**

249

250 *Seed germination assays*

251

252   Osmotic stress caused a general reduction in the percentage of seeds germinated after three weeks of

253 treatment, but with clear quantitative differences between the different populations (Fig. 1). Concerning

254 germination (in water) of control seeds, spruce populations could be divided in two groups: the first one

255 (Valea Mare, Albac, and Paraul Turculet) showed high germination percentages, over 85 %, while in the

256 second one, including the remaining populations, seed germination ranged between 40 % and 50 %. The

257 relative resistance to PEG-6000 of seeds from the different populations did not correspond to germination

258 rates in the control. Under the strongest osmotic stress tested (-0.52 MPa), seeds from the Gioristea-Calimanut

259 and Sudrigiu populations showed the smallest relative inhibition of germination; in GC seeds, in fact,

260 differences in germination percentages were not statistically significant. On the other hand, seeds from Valea

261 Mare and Paraul Turculet were the most affected by PEG-induced osmotic stress (Fig. 1).

262

263 **Fig. 1** Germination rates of seeds from the seven analysed *Picea abies* populations (identification codes as in
264 Tab. 1), after three weeks of osmotic stress treatments; the indicated osmotic pressures were generated by
265 increasing PEG-6000 concentrations. Values are means with SD (n = 3). Different lowercase letters in a
266 column indicate significant differences in germination rates between treatments, for each population.

267 Different capital letters in a row indicate significant differences in germination rates between populations for
268 each treatment according to the Tukey test (α=0.05)
269
270
271   Osmotic stress also affected germination time in seeds from most populations, albeit only slightly.

272 Considering the calculated average values, mean germination time (MGT) generally increased with increasing

273 osmotic stress, but the differences between control and osmotic-stressed seeds were not statistically

274 significant for populations Gioristea-Calimanut, Paraul Turculet and Jepi (Fig. 2).

275

276 **Fig. 2** 'Mean germination time' (MGT) of seeds from the seven analysed *Picea abies* populations
277 (identification codes as in Tab. 1), after three weeks of osmotic stress treatments; the indicated osmotic
278 pressures were generated by increasing PEG-6000 concentrations. Values are means with SD (n = 3).
279 Different lowercase letters in a column indicate significant differences in MGT between treatments, for each
280 population. Different capital letters in a row indicate significant differences in MGT between populations for
281 each treatment according to the Tukey test (α=0.05)
282

283

284   Finally, the 'seedling vigour index' was calculated for all populations and treatments (Fig. 3). Large

285 differences between populations were found for control seeds germinating in water, but in all cases a decrease

286 in SVI values was observed in the presence of PEG-6000, except for the Sudrigiu population, where the SVI

287 remained practically constant. The populations most affected by osmotic stress during seed germination,

288 according to the relative decrease of SVI values, were Valea Mare and Basca Mica (Fig. 3).

289   Summarising, seeds from the Sudrigiu and Gioristea-Calimanut populations showed the smallest

290 decrease of germination percentages and were amongst those with no or only a small increase in MGT. On the

291 other hand, seeds from Valea Mare and Paraul Turculet seemed to be the most affected by PEG-6000. It is

292 interesting to note that the Sudrigiu seeds, apparently the most resistant to osmotic stress, are also those

293 showing the lowest germination rate (40%) and SVI (845), and the highest MGT (14.8 days) of all tested

294 populations (Figs. 1, 2 and 3), in the control; that is, under non-stress conditions.

295

296 **Fig. 3** 'Seedling vigour index' (SVI) of seeds from the seven analysed *Picea abies* populations (identification
297 codes as in Tab. 1), after three weeks of osmotic stress treatments; the indicated osmotic pressures were
298 generated by increasing PEG-6000 concentrations
299

300

301 *Water content percentage in water-stressed Picea abies seedlings*

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303   Water content in the needles of control seedlings ranged between 63 and 72%, without significant

304 differences among the analysed spruce populations, except when comparing those from Jepi and Gioristea-

305 Calimanut, which showed the highest and lowest values, respectively (Fig. 4).Water stress caused a

306 significant decrease in water content in all populations, but seedling dehydration was not uniform. The largest

307 differences within single populations were recorded in the Albac (3.7-fold), Jepi (3.5-fold) and Paraul

10

Turculet (2.7-fold) samples, while the smallest were observed in the populations from Sudrigiu (1.3-fold) and Gioristea- Calimanut (1.4-fold). Therefore, the spruce seedlings from Sudrigiu appeared to be the most resistant to drought-induced dehydration, followed by GC.

Fig. 4 Water content (%) in needles of *Picea abies* seedlings of the seven studied populations after 42 days of water stress treatments. Population identification codes are those defined in Tab. 1. Values shown are means with SD (n=3). Significant differences between populations are indicated by different lowercase Latin letters (for non-stressed control plants) or by different Greek letters (for drought-stressed plants) over the bars, while an asterisk (*) shows significant differences in needle water content between control and water-stressed seedlings within each population, according to the t-test (α=0.05)

Chlorophyll a, chlorophyll b and total carotenoids contents

Needle contents of chlorophyll *a*, chlorophyll *b*, and total carotenoids were determined in seedlings of all *P. abies* populations, in control and drought-stressed plants. In control plants, mean chlorophyll *a* levels in the Basca Mica and Albac populations were significantly lower than those measured in seedlings from Sudrigiu and Paraul Turculet, and intermediate values were determined in the other populations. Water stress treatments induced significant reductions of chlorophyll *a* contents in the Valea Mare, Albac, Paraul Turculet and Jepi populations – the latter showing the largest decrease, by more than 40% relative to the control. Non-significant changes were observed in the Basca Mica population, and small, but significant, increases in chlorophyll *a* levels were determined in the remaining two provenances, Gioristea-Calimanut and Sudrigiu (Fig. 5a).

Absolute chlorophyll *b* contents were lower, about half of those of chlorophyll *a*, but the general patterns of variation between populations and the changes induced by water stress within each population, were similar, except that Jepi seedlings showed a smaller reduction, of 23% relative to the control, as compared to that observed for chlorophyll *a* (Fig. 5b).

Total carotenoids did not differ significantly between populations in control seedlings. However, drought-induced changes in the concentration of these pigments varied in the different spruce populations; significant increases were measured in Gioristea-Calimanut and Sudrigiu, significant decreases in Albac and Jepi, and no significant variation in the other three populations (Fig. 5c).

Fig. 5 Chlorophyll *a*, chlorophyll *b* and total carotenoids contents in needles of *Picea abies* populations after 42 days of water stress treatments. Population identification codes as defined in Tab. 1. Values shown are means with SD (n=3). Significant differences between populations are indicated by different lowercase Latin letters (for non-stressed control plants) or by Greek letters (for drought-stressed plants) over the bars, while an asterisk (*) shows significant differences in needle pigment content between control and water-stressed seedlings within each population, according to the t-test (α=0.05)

Proline and total soluble sugars

349

350   Proline levels in control seedlings varied significantly between populations, in the range of 8 to 30

351   μmol g^{-1} DW, and increased in all cases in response to the drought treatment (Fig. 6a). The largest relative Pro

352   accumulation in relation to the control (8-fold) was observed in Valea Mare seedlings. Spruce plants with

353   origin in Basca Mica, Jepi, Albac and Paraul Turculet showed drought-dependent increases in Pro contents

354   between 3- and 4-fold, while Pro concentration changed very little in Gioristea-Calimanut and Sudrigiu

355   populations (Fig. 6a).

356   Total soluble sugars did not vary between populations, as compared to Pro levels. According to TSS

357   contents in control seedlings, the seven studied populations could be separated in two groups without

358   significant differences within each group: Albac, Paraul Turculet and Jepi, with TSS ranging between 45 and

359   47 mg eq. glucose g^{-1} DW, on the one hand; and Gioristea-Calimanut, Valea Mare, Sudrigiu and Basca Mica

360   (60-65 mg eq. glucose g^{-1} DW), on the other. TSS levels increased in all populations in response to water

361   stress, but only slightly, not even doubling. The largest differences were measured in Paraul Turculet (65%

362   increase) and Jepi (45%) populations, while in Gioristea-Calimanut and Albac seedlings the differences were

363   not statistically significant; TSS increases of 10% to 30% were observed for the remaining provenances (Fig.

364   6b). It should be noted that there was no correlation between the relative changes in Pro and TSS

365   concentrations.

366

367   **Fig. 6** Changes in proline (Pro) and total soluble sugars (TSS) contents in needles of *Picea abies* seedlings
368   after 42 days of water stress treatments. Population identification codes as defined in Tab. 1. Values shown
369   are means with SD (n=3). Significant differences between populations are indicated by different lowercase
370   Latin letters (for non-stressed control plants) or by Greek letters (for drought-stressed plants) over the bars,
371   while an asterisk (*) shows significant differences in needle osmolyte content between control and water-
372   stressed seedlings within each population, according to the t-test (α=0.05)

373

374

375   *Total phenolic compounds and flavonoids*

376   Total phenolic compounds and total flavonoid levels were relatively uniform in control seedlings of

377   most populations, ranging between 10 and 15 mg eq. GA g^{-1} DW, approximately, for TPC (Fig. 7a) or

378   between 8 and 12 mg eq. C g^{-1} DW for TF (Fig. 7b). The exception was the Jepi population for which lower

379   contents of TPC (ca. 7 mg eq. GA g^{-1} DW) and TF (4.4 mg eq. C g^{-1} DW) were determined. These values

380   generally increased in needles of water-stressed spruce seedlings, but quantitative differences varied between

381   populations (Fig.7). The highest relative increases in TPC and TF were observed in Jepi seedlings (2.3-fold

382   and 3.5-fold, respectively), followed by Paraul Turculet and Albac, and also Valea Mare in the case of TF

383   (between 1.4 and 1.7-fold increases). Smaller stress-induced accumulation of antioxidant phenolics, or no

384   significant changes were detected in the other populations (Fig.7). Within each population, the observed

385 patterns of variation of TPC and TF were similar, as should be expected since flavonoids represent the largest

386 subgroup of phenolic compounds.

387

388 **Fig. 7** Changes in total phenolic compounds (TPC) and total flavonoids (TF) in needles of *Picea abies*
389 populations after 42 days of water stress treatments. Population identification codes as defined in Tab. 1.
390 Values shown are means with SD (n=3). Significant differences between populations are indicated by
391 different lowercase Latin letters (for non-stressed control plants) or by Greek letters (for drought-stressed
392 plants) over the bars, while an asterisk (*) shows significant differences in needle TPC or TF content between
393 control and water-stressed seedlings within each population, according to the t-test (α=0.05)

394

395

4. Discussion

397   Drought is a major abiotic stress, with complex effects on plants, inducing a wide range of
398 physiological and biochemical responses which can lead to growth inhibition and, eventually, plant death
399 (Farooq et al. 2009; Harb et al. 2010). Early diagnosis of drought in plants is of critical importance to
400 minimise deleterious effects, as morphological responses to different types of abiotic stress are similar and
401 symptoms specific for water stress are often not easily recognised. In addition, in species of slow growth,
402 such as forest trees, measurable inhibition of growth – which is generally the easiest to detect effect of
403 drought and other abiotic stresses – requires long treatment periods.

404   Climate change scenarios predicted for the next decades will cause a higher exposure of spruce
405 stands to adverse drought conditions, leading to increasing forest stress and mortality (Walker et al. 2015). In
406 the present study drought-induced changes in the levels of several metabolites – which could be employed as
407 biochemical stress markers – have been quantified in needles of spruce seedlings of seven different
408 provenances, subjected to a short, but severe water stress treatment in the greenhouse. As it is well established
409 in many other plant species (Bartels and Sunkar 2005), the general responses to water stress in spruce
410 seedlings included, among others, a decrease in the needles water content, a decrease in chlorophyll *a* and *b*
411 levels, accumulation of osmolytes such as proline and soluble sugars, and an increase in the contents of some
412 non-enzymatic antioxidants, namely total phenolic compounds and flavonoids. The identification of suitable
413 drought stress markers in this species would help in the initial screening of more tolerant populations, but
414 further longer –time experiments are still when extrapolating such results to forest management programmes.
415 Another aspect that should be taken into consideration is that greenhouse conditions do not overlap with
416 natural conditions of forest stands. For instance in this study CO_2 concentration is around 100 ppm under the
417 present conditions (Earth's CO_2 Home Page) and therefore such results obtained should be reinforced with
418 outdoor experiments in order to guide adaptive management and tree improvement.

419   Drought causes cellular dehydration in plants (Hoekstra et al. 2001; Morgan 1984; Toldi et al. 2009),
420 and there are many publications reporting the loss of water in the leaves of different species, in response to
421 water stress (e.g., Munné-Bosch and Peñuelas 2004; Saura-Mas and Lloret 2007; Yang et al. 2010). Changes

13

422 in spruce water content have been previously investigated in trees of different ages and in different tissues,
423 showing large variations in phloem water content but not so big differences in heartwood water (Gall et al.
424 2002; Kravka et al. 1999). Drought damages are observed mostly in spruce seedlings and young trees; the
425 physiological processes triggered by water stress include a drop in tissue water and stomata closure, with the
426 subsequent inhibition of photosynthesis, changes that become irreversible above a water deficit of 33%
427 (Mejnartowicz and Lewandowski 2007). The response of white spruce [*Picea glauca* (Moench) Voss] or
428 black spruce [*Picea mariana* (Mill) B.S.P.] seedlings to water stress treatments indicated a significant
429 reduction in water content, even after a drought period shorter than that used in the present study (Marshall et
430 al. 2000); this suggests that water content or, more specifically, the decrease in water content, can be
431 considered as a suitable physiological water stress marker in this genus. Yet, while a drop of water content in
432 the needles of *P. abies* seedlings was observed in all analysed populations, some of them were clearly less
433 affected, such as those from Gioristea-Calimanut and, especially, from Sudrigiu. The capacity to reduce
434 drought-induced needle dehydration could provide an indication of the relative degree of drought tolerance of
435 the investigated populations, at least at the seedling stage.

436   A decrease in the levels of photosynthetic pigments due to drought is a common physiological
437 response in many plant species (Al Hassan et al. 2017; Lei et al. 2006), including conifers (Alonso et al. 2001;
438 Miron and Sumalan 2015) The observed effects of water stress on spruce seedlings pointed to a slight
439 reduction in photosynthetic activity in stressed seedlings, due to a general degradation of chlorophyll *a* and
440 chlorophyll *b*. Yet some populations appeared to be less affected than the rest to degradation of
441 photosynthetic pigments under drought conditions including, here again, spruce seedlings derived from
442 Gioristea-Calimanut and Sudrigiu.

443   Proline (Pro) accumulation in plants in response to water deficit is a quite general phenomenon,
444 which has been previously reported in many taxa, including conifers; in some species, the relative increase in
445 Pro levels in relation to the non-stressed control can reach 100-fold or even more (Al Hassan et al. 2016a, d;
446 Cyr et al. 1990; Heuer 2010; Pardo-Domènech et al. 2015; Patel and Vora 1985;). As in other studies, a
447 significant increase of Pro in Norway spruce seedlings was reported after a short time of severe water stress
448 treatments (Ditmarová et al. 2010), highlighting the potential of measuring drought-induced changes in Pro
449 contents well before any growth inhibition can be detected in this slow-growing species. Yet there is not a
450 clear, general correlation of Pro levels with the degree of stress tolerance, which varies widely in different
451 taxa. Previous comparative studies have established a positive correlation between drought tolerance and Pro
452 accumulation in some species (Jiménez et al. 2013; Lei et al. 2006), whereas in others this correlation is
453 negative (Al Hassan et al. 2016c; Silvente et al. 2012) or no correlation could be found (e.g. Bhaskaran et al.
454 1985). This means that Pro can either be directly involved in the mechanisms of drought tolerance or simply
455 act as an indicator of the level of stress affecting the plants. In *P. abies*, the latter possibility seems to apply,
456 since the lowest Pro accumulation under water stress conditions was observed in seedlings from the Sudrigiu
457 population, which according to other biomarkers appears to be the less affected by drought, as discussed
458 above.

14

459   Soluble sugars are also common omolytes in plants, and it is known that they can also accumulate in

460   needles, sapwood or inner bark as a response to the drought-induced lowering of the osmotic potential

461   (Clancy et al. 1995). Moreover, numerous greenhouse studies in which plants are subjected to water stress

462   treatments have revealed a significant increase of soluble sugars contents in leaves (Al Hassan et al. 2016b, c;

463   Jiménez et al. 2013; Sudachkova et al. 2002; Tan et al. 1992). The same pattern has been observed in the

464   present study, although the drought-induced increases in soluble sugars are relatively small. In addition, due

465   to their multiple biological roles as direct products of photosynthesis, components of primary metabolism,

466   precursors of other compounds and even signalling molecules, it is very difficult to assess the specific

467   functions of soluble carbohydrates in the mechanisms of stress tolerance (see Gil et al. 2013, for a more

468   extensive discussion on this topic). Therefore, total soluble sugars may not be reliable biochemical markers of

469   drought stress in Norway spruce.

470   The activation of antioxidant systems, both enzymatic and non-enzymatic, is also a general response

471   to drought and other abiotic stresses, which cause oxidative stress in plants as a secondary effect. Phenolic

472   compounds and, within them, the subgroup of flavonoids contain strong antioxidant molecules; there is

473   overwhelming evidence that these 'secondary metabolites' play a major role in the mechanisms of defence of

474   plants against environmental stresses, including drought (Ramakrishna and Ravishanka 2011). In some

475   populations of *P. abies* seedlings, total phenolics and antioxidant flavonoids increased significantly in

476   response to water stress, as has been reported for many other species (e.g., Al Hassan et al. 2016a; Bautista et

477   al. 2016; Hernández et al. 2004). However, this pattern was not uniform as some spruce populations –

478   including Gioristea-Calimanut and Sudrigiu – showed very small increases or non-significant changes in TPC

479   and TF levels, suggesting that they could be relatively less affected by drought-induced oxidative stress.

480   Despite the variability in the patterns of physiological and biochemical parameters detected in the

481   different *P. abies* populations, in response to water stress, the simultaneous assessment of several of these

482   putative biomarkers could provide relevant information on the deleterious effects of drought on spruce

483   seedlings, well before growth inhibition can be observed. In practice, the most reliable drought stress

484   biomarkers in *P. abies* appear to be the water content, chlorophylls and proline levels in the needles. These

485   parameters can be easily and rapidly determined, using simple methods that require small amounts of plant

486   material.

487   The use of this battery of biomarkers clearly points to the spruce seedlings with origin in Sudrigiu,

488   followed by those from Gioristea-Calimanut, as the most resistant to drought: lowest reduction in water

489   content (i.e, highest resistance to drought-induced dehydration), no significant degradation of chlorophylls,

490   and lowest proline accumulation. This conclusion is strongly supported by the results of complementary

491   experiments in which seeds of the same stocks were germinated *in vitro* in the presence of PEG-6000.

492   According to different parameters of germination, such as germination rates, mean germination time (MGT)

493   and 'seedling vigour index' (SVI), these assays revealed that the same populations, Sudrigiu and Gioristea-

494   Calimanut, are also the most tolerant to osmotic stress during seed germination.

495　　　　The climatic characteristics of the geographical locations of these two spruce populations may
496　explain a relatively higher drought tolerance. Sudrigiu is located at the lowest altitude of all selected
497　provenances (230 m), which correlated with the highest mean annual temperature and highest potential
498　evapotranspiration. Gioristea-Calimanut is located at higher altitude, and consequently has a lower mean
499　annual temperature, but is the population affected by the lowest mean annual precipitation. Therefore, the
500　slightly different responses to water stress of the Sudrigiu and Gioristea-Calimanut seedlings, as compared to
501　the rest, could be due to adaptation of the original trees to somewhat drier natural environments.

502　　　　As mentioned above, the short water stress treatments applied did not allow detecting inhibition of
503　seedling growth, and the association of the selected biomarkers with plant performance under stress has not
504　been directly demonstrated – although it is clearly established for many other species. Therefore, the results
505　presented here should be confirmed and extended in future studies involving longer drought treatments and
506　determination of their effect on growth parameters. Moreover, we do not know yet whether these biomarkers
507　are also suitable to assess drought responses in older trees; this is likely, considering that seed germination
508　and early seedling growth are generally more sensitive to stress than later developmental stages, but
509　additional experiments will be required to confirm it. Suitable biochemical markers could be used for a rapid
510　initial screening of a large number of individuals from different populations, but other functional traits, such
511　as rate of growth or productivity should be taken also in consideration in the selection of the optimal
512　genotypes in reforestation programmes.

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