1 Practical implications of different phenotypic and molecular responses of evergreen

2 conifer and broadleaf deciduous forest tree species to regulated water deficit in a

3 container nursery

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35 **Abstract**

36 Recent climatic changes have resulted in an increased frequency and prolonged periods of

37 drought and strained water resources affecting plant production. We explored the possibility of

38 reducing irrigation in a container nursery and studied the growth response of seedlings of

39 economically important forest trees: broadleaf deciduous angiosperms *Fagus sylvatica*,

40 *Quercus petraea* and evergreen conifers *Abies alba* and *Pinus sylvestris.* We also studied

41 markers of water stress including modifications of biomass allocation, leaf anatomy, proline

42 accumulation and expression of selected genes. Growth of the broadleaved deciduous species

43 was more sensitive to the reduced water supply than that of conifers. Remarkably, growth of

44 the shade tolerant *Abies* was not affected. Adjustment of biomass allocations was strongest in

45 *P. sylvestris,* with a remarkable increase in allocation to roots. In response to water deficit both

46 deciduous species accumulated proline in leaves and produced leaves with shorter palisade cells,

47 reduced vascular tissues and smaller conduit diameters, but not conifers. Relative transcript

48 abundance of a gene encoding a Zn-finger protein in *Q. petraea* and a gene encoding a pore

49 calcium channel protein 1 in *A. alba* increased as water deficit increased. These findings suggest

50 that in container nursery, the genetic selection can be initiated by water deficit. Our study shows

51 major differences between functional groups in response to irrigation, with seedlings of

52 evergreen conifers having higher tolerance than the deciduous species. This suggests that major

53 water savings could be achieved by adjusting irrigation regime to functional group or species

54 requirements.

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56 Key words: biomass allocation; drought; irrigation; leaf anatomy; mRNA level; proline

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Introduction

According to predictions, global warming will lead to an increased frequency and extent of summer drought periods in many regions of the Earth [1]. Although in northern and western European forests global warming is expected to stimulate forest growth, the accompanying drought may cause adverse effects [2]. This is because climate warming will increase evapotranspiration more than precipitation and periodic drought events will enhance effects of increased temperature on growth and photosynthesis of boreal and temperate tree species [3,4]. The understanding of drought effects on trees is thus vital for proper management and conservation of forest ecosystems.

Water shortages affect all biological functions of trees, with the risk of drought-induced mortality depending on the local geographical conditions, precipitation, soil properties and anthropogenic factors [5]. Vulnerabilities of individual species vary widely [6,7,8,9]. Much knowledge of the variability of tree responses to drought has been gathered from studies of mature stands [10,11,12], however, the most vulnerable stage in the tree's ontogeny is the seedling. In particular, it is not certain to what extent the responses and vulnerabilities of adult trees vs. seedlings to drought are consistent in between-species comparisons [13,14].

Trees acclimate to restriction in water supply through modifications of growth, biomass allocation, organ structure, and through functional adjustments encompassing processes from gene expression to enzymatic activity and metabolic regulation [15,7,16,17]. These adaptive changes may lead to improved plant survival through an increased water uptake and a more conservative water use [18]. Water deficit typically leads to an increase in relative biomass allocation to roots, a decrease in allocation to leaves and reduced leaf area ratio and leaf mass fraction, resulting in greater water uptake potential and reduced transpiration [19,20,21]. Individual leaves produced under the influence of drought are usually smaller and thicker than those from well hydrated plants [22,23,24] and show reduced amount of vascular tissues and smaller diameters of conductive elements [25,26,27]. Plants subjected to drought may accumulate metabolites responsible for osmotic adjustment and oxidative stress protection as well as change hormone and regulatory molecule concentrations [28,29,30]. Profiles of gene expression and enzymatic as well as transport activities may become greatly altered [31,32]. Adjustments occur at different time scales, but ultimately may result in drought resistant or drought tolerant phenotypes. The ability to produce such plastic modification may be the key to plant survival under natural settings. However, traits that enhance drought survival, especially those related to water conservation, frequently trade off with growth potential [33].

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101 For example, increased allocation to roots may lead to a decreased ratio of assimilatory to non-
102 assimilatory biomass, restricting whole-plant carbon supply even after the cessation of drought
103 [34]. Moreover, long term carry-over effects (so called drought memory) may also result from
104 metabolic adjustment to drought [35,36].

105   Water requirements and responses to drought differ significantly among taxa and
106 functional groups. For major forest tree species the grouping into drought tolerance categories
107 or rankings has been based on characteristics of their habitat and responses of growth and
108 physiological indices to environmental drought [6,7]. In general, tree species adapted to drier
109 environments are more tolerant to drought compared with those from humid habitats [37,38].
110 At the functional group level, evergreen conifers are more tolerant to drought than deciduous
111 broadleaved tree species [6] and late successional, shade tolerant tree species are often more
112 sensitive to water deficit than pioneer and shade intolerant species [39]. Many insights have
113 been provided by physiological studies, especially those focusing on vulnerability to cavitation
114 and responses of stomatal conductance [7,40]. Such results may provide general information
115 on species requirements and drought vulnerability ranking, yet, since they focus on saplings or
116 adult trees, their relevance for seedling stages may be limited.

117   The species selected for this study were two deciduous broadleaf angiosperms (*Fagus*
118 *sylvatica* L. and *Quercus petraea* (Matt.) Liebl. and two evergreen conifers (*Abies alba* Mill.
119 and *Pinus sylvestris* L.). They differ in ecological requirements with respect to moisture and
120 their overall sensitivity to water deficit has been investigated under various settings. Briefly,
121 *Fagus sylvatica* is a dominant European forest tree species of high economic and ecological
122 importance. It is a shade-tolerant, late successional and the most water-demanding tree among
123 our selected species [41,7]. It requires high air humidity and grows preferentially on fertile,
124 well-drained and moist, but not waterlogged soils. *Quercus petraea* is a moderately shade
125 tolerant, late successional tree. It maintains higher leaf conductance [42] and higher net CO_2
126 assimilation rates than *F. sylvatica* under the same experimental conditions [43,44]. *Abies alba*
127 is a strongly shade tolerant, late successional and slow-growing tree [45]. It is considered rather
128 sensitive to drought [46,47]. *Pinus sylvestris* is the most widely distributed Eurasian conifer. It
129 is a pioneer, early-successional, light-demanding and drought-tolerant tree. Severe drought,
130 however, has a limiting effect on *P. sylvestris* growth and may triggered Scots pine decline [48].
131 It may be expected that species' growth sensitivity to low water supply should follow ecological
132 species ranking [41]. Our study species can be classified according to their decreasing drought
133 tolerance in the following order: *P. sylvestris > A. alba > Q. petraea > F. sylvatica*. However,

134   this ranking cannot be generalized to seedlings and to the whole geographical ranges of these
135   species. In addition, it is not clear whether seedlings of our study species growing in a container
136   nursery will show similar drought tolerance as in natural conditions.

137   Raising seedlings under polyethylene tunnel has become an important method of
138   containerized forest tree production [49]. Usually, seedlings are grown in containers placed
139   under an unheated tunnel. Under Central European conditions, seed planting takes place in May
140   and seedlings may be grown till the end of July, August or September, depending on the species,
141   and then the seedlings are transferred to an outdoor nursery. Nutrients are provided with water
142   used for irrigation or as slow-released fertilizers added to the substrate. Irrigation in container
143   forest nurseries is usually carried out by programmable automatic systems delivering water
144   according to established norms [50]. The rising cost of water provides incentive to reduce water
145   consumption, however decreasing irrigation doses below physiological optimum may result in
146   water stress and decrease the development rate of seedlings. On the other hand, reduced
147   substrate water content may improve substrate aeration which is beneficial for growth and the
148   quality of seedlings [51] and reduce nutrient leaching from the substrate [52]. Lower substrate
149   water content, especially at the end of the growing season, accelerates tissue maturation and
150   bud set, resulting in improved frost tolerance [53]. These additional benefits of reduced
151   irrigation thus occur in addition to water savings.

152   In this study we evaluated the effects of reduced irrigation on growth and on several
153   morphological, anatomical, metabolic and molecular variables that are known to predictably
154   respond to water deficit. We studied whole plant allocation of biomass among major organ
155   types (leaves, roots and stems), examined some key anatomical dimensions of foliar cross-
156   section, and determined the concentration of proline – a compound involved in the process of
157   osmotic adjustment [54,55]. Additionally, we studied the expression of genes known to respond
158   to water deficit in our focal species. Although the molecular basis of phenotypic responses of
159   forest trees to water deficit is not well understood, and the identification of water deficit related
160   genes in trees, especially in conifers, has been difficult, partly because of their large genomes
161   [56], sets of such reference drought-responsive genes have been identified first in *Arabidopsis*
162   and some crops [57], and later in other plants, including trees [58,59]. These genes may be
163   considered as indicators of drought stress [60].

164   We tested the hypotheses that under decreased irrigation water supply: (1) seedling
165   growth will be reduced and the reduction of biomass accumulation will be relatively larger in
166   broadleaved deciduous and shade-tolerant species than in conifers and light-demanding species;

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167   (2) allocation to roots will increase and allocation to leaves will decrease; (3) leaves will show

168   xeromorphic anatomical adjustments and (4) proline accumulation will be enhanced; (5)

169   relative levels of mRNA of stress-related genes in leaves will be elevated. The additional,

170   applied facet of our study was to test whether, and in which species, irrigation doses can be

171   reduced below the established norm without compromising growth and affecting morphological,

172   metabolic and molecular characteristics of the seedlings.

173

174   **Material and methods**

175   Study site

176   The experiment was conducted in the Rogoziniec forest nursery (N52°18' E15°46') located

177   in Babimost Forest Division , SW Poland. The experiment was set up in an unheated

178   polyethylene tunnel (8 x 50 m).

179

180   Plant material

181   *Fagus sylvatica* seeds and *Quercus petraea* acorns originated from the seed stands located

182   close to Swiebodzin (52°15' N, 15°32' E), *Pinus sylvestris* seeds were from the local

183   'Rogoziniec' provenance (52°10' N, 15°50' E) and *Abies alba* seeds originated from the

184   Sudety Foothills (51°7' N, 15°55' E), Poland.

185

186   Experimental design

187   All seeds were subjected to stratification. At the end of May 2015 they were sown into

188   individual compartments of 54-compartment polystyrene containers (Robin, France). Each

189   compartment was filled with 430 ml of substrate consisting of peat and perlite (3:1 v/v, *pH* =

190   6.5) with the addition of 2 g of the 'Osmocote Exact Standard' slow-release fertilizer

191   (N:P:K:Mg 15:9:12:2, with micronutrients). During a ten-day germination period containers

192   were watered every day up to the field capacity. Containers were placed on benches 0.30 m

193   above the ground and arranged in a full-factorial completely randomized block design with

194   species and irrigation levels as the fixed factors and blocks as the random factor. In our

195   experiment, the irrigation norms recommended by Pierzgalski et al. [61] for forest nurseries

196   in Poland were used as the benchmark. In these guidelines, the species of trees are classified

197   into one of three groups: (1) conifers including *Pinus sylvestris* and *Abies alba* with one

198   deciduous species *Tilia cordata*, (2) *Quercus*, *Fagus sylvatica* and *Tilia platyphyllos*, (3) other

199   broadleaved deciduous species. From April to June, according to these norms, at the initial

6

200   phase of growth, seedlings are irrigated every two days with 5 mm for *P. sylvestris* and *A.*

201   *alba* and with 7 mm every three days for the broadleaved deciduous species. In our

202   experiment, we had regard for the norms and nurseryman' experience. Four daily irrigation

203   levels were established: 6 mm (6 l/m^{-2}, 100%), 4.5 mm (4.5 l/m^{-2}, 75%), 3 mm (3 l/m^{-2}, 50%)

204   and 1.5 mm (1.5 l/m^{-2}, 25% of the maximal dose). When recalculated per area of one cavity of

205   container, 12 (100%), 9 (75%), 6 (50%) or 3 ml (25%) of water were delivered every day.

206   After leaf expansion, water amount reaching the substrate in cavity was reduced when

207   compared to these values due to interception. To compare species-specific responses to the

208   irrigation doses, we applied the same irrigation regime for each species. Each irrigation

209   regime was replicated four times with four containers per species and irrigation treatment per

210   block (4 blocks x 4 species x 4 treatments x 4 containers x 54 cavities). At the end of the

211   experiment, the total number of seedlings was 8008. Seedlings were randomly selected, and

212   the numbers of replicates per each analysis (*n*) are given in the Results. Additional containers

213   with seedlings of Scots pine were placed around the experimental blocks to eliminate the edge

214   effect.

215

216   Microclimate

217   Air temperature and relative humidity (*RH*) in the tunnel were monitored with two HOBO Pro v2

218   probes (*OnSet Computers*, Pocasset, MA, USA). The probes were attached at the height of 90 cm

219   above the ground (close to the apical parts of seedlings) and logged data every twenty min.

220   During the active growing phase from mid-June to the beginning of August mean air

221   temperature ($T_{mean}\pm$SD, where SD is standard deviation) was 23.4$\pm$7.4 °C and the average

222   relative humidity ($RH_{mean}\pm$SD) was 61$\pm$22 %. In July $T_{mean} = 20.7\pm6.0$ °C, maximal

223   temperature (T_{max}) was 38.8 °C, minimal temperature (T_{min}) was 10.8 °C, and $RH_{mean} =$

224   65$\pm$19%, RH_{max} = 96 %, and $RH_{min} = 28$ %. In August $T_{mean} = 24.2\pm7.6$ °C, $T_{max} = 42.5$ °C,

225   $T_{min} = 7.1$ °C, and $RH_{mean} = 60\pm23$ %, $RH_{max} = 96$ %, and $RH_{min} = 19$ %. Extremely high

226   temperatures exceeding 40 °C occurred on 6 and 8 August between 2:00 and 4:30 p.m.

227

228   Regulated water deficit

229   The terms water deficit and drought have often been considered synonyms although water

230   deficit is more commonly used when referring to water availability below field capacity,

231   especially in relation to crop cultivation whereas drought is generally related to low

232   precipitation over a period of time. In our study, seedlings were cultivated under controlled

233   conditions in an unheated tunnel, therefore we use the term 'water deficit' for irrigation levels

234   below the established norm.

235   Plants were watered by an overhead sprinkler system. The different irrigation levels

236   were obtained by regulating the duration of water delivery over particular blocks. The water

237   pressure (0.25 MPa in each sprinkler) and irrigation time were monitored using an automated

238   system (Rathmakers, Germany). A pluviometer installed below the overhead sprinklers were

239   used to measure water amount in millimeters in each irrigation treatment.

240

241   Growth, biomass allocation and water contents

242   The height and root collar diameter of seedling were measured after 60 days of growth under

243   experimental conditions ($n = 10$, n – number of seedlings per block, species and per

244   treatment). Seedlings were subsequently removed from containers and their roots were gently

245   washed ($n = 4$). Plants were dissected into roots, stem and leaves and fresh mass (FM) of each

246   fraction was determined. Biomass fractions were then dried at 65ºC for 7 days until the weight

247   was unchanged using a climate cabinet (Pol-Eko, Poland), and weighed for dry mass

248   determination. Water amount in each type of organs was calculated by subtracting organ dry

249   mass from its fresh mass and water content per unit of dry mass was obtained by dividing the

250   water amount by dry mass of the organ fraction or by total seedling dry mass.

251

252   Microscopic observations

253   On 13 August 2015 one mature, undamaged leaf per seedling was sampled from eight

254   seedlings (two from each block) in 25%, 50% and 100% irrigation regimes. We expected that

255   anatomical acclimation in needles could be observed among extremely different irrigation

256   treatments, therefore the 75% irrigation treatment was not considered in the microscopic leaf

257   variables. A 2 mm wide segment from the central part of the needle or the broadleaf lamina

258   (including midrib) was excised and fixed by vacuum infiltration for 2 hours in a mixture of

259   paraformaldehyde (2%) and glutaraldehyde (2%) followed by overnight incubation under

260   atmospheric pressure. Samples were dehydrated in graded ethanol series. *Abies, Fagus* and

261   *Quercus* samples were embedded in Technovit resin (Heraeus Kulzer GmbH, Germany),

262   sectioned to 5 µm with a microtome (Leica RM 2265, Leica Biosystems, Germany) and

263   stained with 0.01% solution of toluidine blue in 1% sodium borate. Sections of *Pinus* needles

264   (35 µm thick) were obtained using a VT 1200 S vibratome (Leica, Germany). Micrographs

265   were taken using a light microscope (Axioscope A1, Zeiss, Germany) with an attached digital

266 camera (AxioCam MRC5, Zeiss). Micrometric traits were measured with Axiovision 4.9.1

267 (Zeiss, Germany). In each leaf we determined the thickness of lamina, the length of palisade

268 cells (mean of 3-5 cells per leaf, except in *Pinus* where palisade mesophyll does not occur),

269 transverse area of xylem and phloem in the central vein, and mean and maximal diameter of

270 conduits (based on 10 widest vessels in the angiosperms and tracheids in the conifer species).

271

272 Determination of leaf proline

273 Proline concentration was determined with the method of Bates et al. [62]. Fully expanded

274 leaves from the apical portion of the seedlings were sampled on 7 August 2015. Purified

275 proline was used to standardize the procedure for quantifying sample values. Acid-ninhydrin

276 was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M

277 phosphoric acid at the temperature 75°C until dissolved. A 0.3 g sample of plant material was

278 homogenized in 3 ml of 5% aqueous sulfosalicylic acid and the homogenate filtered through

279 Whatman # 2 filter paper. A 2 ml aliquot of filtrate was incubated with 2 ml of acid-ninhidrin

280 and 2 ml of glacial acetic acid in a test tube for 1 hour at 100°C, and the reaction was

281 terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene by mixing

282 with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from

283 the aqueous phase, warmed to room temperature and the absorbance read at $\lambda = 515$ nm

284 (Spekol, CarlZeiss, Jena) using toluene as blank. The proline concentration was determined

285 from a standard curve and calculated on a dry mass basis using the formula $S_k = K \times A \times T$,

286 where: K is coefficient calculated with the standard curve, A is absorbance and T is

287 conversion factor accounting for the volume of toluene. Proline leaf concentration was

288 expressed in $\mu g\ g^{-1}$ DM.

289

290 Expression of stress-related genes

291   Leaf samples for RNA extraction were collected on 7 August 2015. Leaves without

292 symptoms of necrosis or other visible damage were collected. In each species five seedlings per

293 block and treatment were sampled and three samples were used for analyses. Leaf material was

294 immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

295   Plant material was ground in liquid nitrogen and total RNA was extracted with 2% B-

296 mercaptoethanol in the extraction buffer as described by Chang et al. [63]. Plant material was

297 ground in liquid nitrogen and total RNA was extracted with 2% B-mercaptoethanol in the

298 extraction buffer as described by Chang et al. [63]. The isolated RNA was dissolved in 30 µl

9

299   ddH$_2$O. The extracted RNA was quantified using the Qubit®RNA BR Assay kit (Life

300   Technologies, Carlsbad, CA, USA). The quality of the RNA was checked on a 1% agarose

301   gel. One microgram of RNA per sample was treated with DNase RQ (Life Technologies) and

302   used for cDNA synthesis (Transcriptor First Strand cDNA Synthesis Kit, Roche). The

303   resulting cDNA was diluted 1:10 and used for quantitative real-time PCR in a LightCycler 96

304   (Roche, Manheim, Germany). To each well 2.5 µl of diluted cDNA, 10 µl of SYBR Green I

305   Master (Roche), 1.25 µl of 5 µM mixture of the forward and reverse primers and 5 µl of

306   ddH$_2$O were added for the amplification reaction. Each biological sample for each gene was

307   run twice. The PCR programs were as follows: 5 min at 95°C, 45 cycles of amplification for

308   10 s at 95°C, 10 s at primer annealing temperature (Supplementary Table 1), and 20 s at 72°C.

309   Specificity of PCR amplification was analyzed by melting curve and peaks (60 – 95°C, with

310   temperature ramp 2.2°C per s) and verified on agarose gel.

311   After the RT-qPCR procedure was complete, the quantification cycles were determined

312   using the second derivative maximum method implemented in the Roche LigthCycler 96. The

313   primer efficiency was determined by a dilution series and ranged between 1.8 and 2.0 for the

314   genes of this study. The relative expression was analyzed with $\Delta\Delta C_T$ [64]. The potential

315   reference genes were tested for their expression stability among the all treatment groups using

316   Normfinder [65] implemented in the RefFindr [66]. Gene expression was normalized by the

317   gene expression derived from housekeeping genes: Ubiquitin (*UBQ*, GenBank: AF461687) for

318   *P. sylvestris,* Cyclophilin 2 (*CYC*, GenBank: ERP001867) for *Q. robur*, actin (*ACT*, GenBank:

319   AM063027) for *F. sylvatica* and translation initiation factor IF-2 subunit alpha (*TIF2A,*) for *A.*

320   *alba* (Supplementary Table 1). Real-time quantitative RT-PCR (qRT-PCR) was used to

321   describe plants responses to water deficit. On the base of published data, we selected drought-

322   related genes which were previously found by others to be related to drought stress response

323   (up-regulated or down-regulated) or to abiotic stress in analyzed species. In *F. sylvatica*,

324   abscisic acid (*ABA*)-related drought signaling genes were analyzed: *NCED1* - e9-cis-epoxy-

325   dioxygenase - required for *ABA* biosynthesis (GenBank: DQ787262); *PP2C* - protein phosphate

326   2C - involved in *ABA* signal transduction (GenBank: AJ277743); *ERD10* - early responsive to

327   dehydratation, an *ABA*-responsive transcription factor that attenuates *ABA* responses (GenBank:

328   FR775803); stress protection *APX1* - ascorbate peroxidase genes encoding enzymes required

329   for the scavenging of superoxide radicals, hydrogen peroxide and toxic aldehydes (GenBank:

330   FR774767) [67]. In *Q. petraea* the following genes expression was investigated: α-tubulin

331   (*TUB*) related with actively dividing tissues; Zn-finger protein of *Arabidopsis thaliana* (*ZAT11*)

332   – a gene involved in oxidative stress-induced programmed cell death; glutathione-s-transferase

333   (*GST*) and used primers were previously published by Makela et al. [68]. In *P. sylvestris*: genes

334   involved in biosynthesis (*SPDS* - spermidine synthase, GenBank: HM236827) and metabolism

335   of polyamines (*PAO* – polyamine oxidase, GenBank: HM236830); catalase (*CAT,* GenBank:

336   EU513163), involved in reactive oxygen species homeostasis regulation; pyruvate

337   decarboxylase (*PDC*) (GenBank: CO161777.1) marker of alcohol fermentation in tissues under

338   hypoxia; late-embryogenesis abundant protein (*LEA*), (GenBank: AAX68990.1) the well-

339   known osmoprotectors; glutamate-cysteine ligase (*GCL*), (GenBank: AJ132540.1);

340   glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (GenBank: L07501) [69]. In *A. alba*:

341   genes classified as early responsive to dehydratation (*ERD10*) (GenBank: P42759);

342   chloroplastic beta-amylase 1, b isoform (*BAM1*.b) - (GenBank: Q9LIR6); two pore calcium

343   channel protein 1 *TPC1* (GenBank: AT4G03560) involved in stomatal closure and abiotic stress

344   response; xyloglucan endotransglucosylase/hydrolase (*XTH7,* GenBank: Q8LER3) related with

345   cell growth; one of putative uncharacterized protein (*PUP1*.a10.13.9) (GenBank: A9NLY4)

346   belongs to dehydrin family [58].

347

348   Statistical analyses

349   Prior to analyses, the data were tested for normality and homogeneity of variance in groups

350   with Shapiro-Wilk's and Levene's test, respectively. Data were $\log_{10}$ transformed to fulfill the

351   ANOVA conditions. One-way ANOVA with irrigation regimes as the fixed factor and blocks

352   as the random factor was applied to compare the irrigation treatments within each species for

353   water contents in organs, whole plant dry mass, biomass allocation and gene expression or two-

354   way ANOVA with species and irrigation regimes as fixed factors for leaf proline concentration

355   and anatomical leaf traits at $P < 0.05$. When ANOVA showed significant differences, the mean

356   values were compared with the analysis of contrasts at $P < 0.05$.

357

358   **Results**

359   Seedling water status

360   At time of harvest both gymnosperm species (*Abies* and *Pinus*) had higher LWC (leaf water

361   content) and lower RWC (root water content) than the angiosperm species (*Fagus* and *Quercus*)

362   whereas their SWC (stem water content) were similar (Fig. 1). Lower irrigation resulted in

363   slightly reduced LWC in *Quercus* and *Pinus* although most pairwise contrasts were non-

364   significant (Fig. 1 a). There were also slight decreases of RWC in *Fagus* and SWC in *Quercus*

365 and *Abies* due to reduced irrigation (Fig. 1 b, c). Thus, the measurement of water contents

366 indicated only moderate organ-level water stress.

367

368 Growth

369 Reduction in water availability resulted in decreased height and root collar diameter of *Fagus*,

370 *Quercus* and *Pinus* seedlings while in *Abies* the growth response to the different irrigation

371 regimes was not significant (Table 1). The magnitude of response was largest in *Fagus* (2.2 and

372 1.8-fold reduction in respectively, height and diameter), followed by *Quercus* (1.8 and 1.5-fold

373 reduction and *Pinus* (1.5 and 1.2 fold). Significant height reductions occurred as irrigation was

374 decreased from 100% to 75% (*Fagus* and *Quercus)* and from 75% to 50% and from 50% to 25%

375 of the recommended dose (all three species). Mean slenderness indices (h/d) decreased with

376 reduced irrigation in *Fagus, Quercus* and *Pinus* but not in *Abies* (Table 1).

377   Biomass accumulation differed significantly among species, with seedlings of the two

378 angiosperms *Fagus* and *Quercus* reaching over 2.5 g dry mass under full irrigation, in contrast

379 to the gymnosperms *Abies* and *Pinus* that reached only, respectively, 0.1 and 0.4 g (Table 2;

380 Fig. 2a, b). Irrigation regime affected seedling growth in a dose-response manner in three out

381 the four species (Fig. 2). Under 25% irrigation, dry mass of *Fagus* seedlings was over 5 times

382 smaller than under the 100% dose, whereas in *Quercus* the difference was about three-fold (Fig.

383 2a). There was, however no effect of varied irrigation on the biomass of *Abies* and the biomass

384 decrease between 100% and 25% doses in *Pinus* was approximately by 1/3 (Fig. 2b).

385

386 Allocation

387 Alteration of biomass allocation in response to reduced irrigation was species-specific (Fig. 3

388 a-d). The strongest response was observed in *Pinus,* in which ABMR (aboveground to

389 belowground mass ratio) strongly declined with irrigation reduction from 75 to 50%, resulting

390 from an increased RMF (root mass fraction) and decreases in both LMF (leaf mass fraction)

391 and SMF (stem mass fraction). In contrast, allocation in *Abies* was not affected by irrigation. In

392 *Fagus,* reduced irrigation resulted in increased LMF and decreased SMF without effect at the

393 root or ABMR level. Finally, in *Quercus* only a minor decrease in ABMR was noted at

394 irrigation reduction from 100 to 75%, with variability in organ-level allocation not unaccounted

395 for by irrigation.

396

397 Leaf proline concentration

398   Under 100% irrigation leaf proline concentration was similar across all species ($F_{3, 12} = 1.9$, P
399   $= 0.183$) (Fig. 4). Decline in irrigation resulted in an increase in proline concentration in the
400   two angiosperms but not in the gymnosperm species. Specifically, the increased levels of
401   proline were observed at 50 and 25% dose in *Fagus* and at 75% and less in *Quercus*. The
402   magnitude of proline increase due to irrigation reduction was about two-fold in *Fagus* and
403   somewhat less in *Quercus*.

404

405   Anatomical leaf traits
406   Anatomical leaf traits were analyzed in seedlings exposed to three irrigation regimes: 25, 50 or
407   100% (Fig. 5). We found evidence for acclimation of some anatomical traits in *Fagus* and
408   *Quercus*. Reduction of irrigation resulted in lower leaf lamina thickness in *Quercus*, and
409   shortening of the palisade cells in *Fagus* and *Quercus* (with only a single palisade layer present;
410   Fig. 5 a, b). Both angiosperms also showed reduced development of vascular tissues, with lower
411   cross-sectional areas of xylem and phloem (Fig. 5 c, d) and their vessels exhibited smaller
412   conduits (Fig. 5 e, f). The differences typically occurred between 100% and 50% but not
413   between 50 and 25% irrigation levels. In contrast, none of the above traits varied among
414   treatments in the two conifer species.

415

416   The expression levels of selected stress-response genes
417   In *F. sylvatica*, all transcripts of the analyzed genes were present in leaf samples, but their
418   relative level of gene expression was not affected by treatment ($P > 0.05$). In *Q. petraea*,
419   efficiency of primers for *TUB* was more than 2.0, therefore this transcript was not analyzed. An
420   increase in relative gene expression of *ZAT11* with a decrease in water availability was observed
421   ($r = 0.7059$, $F = 9.9341$, $P = 0.0103$), whereas the relative level of gene expression for *GST* was
422   not affected ($P > 0.05$). In *Pinus sylvestris*, similarly as in *Fagus*, all transcripts were present in
423   all samples, but the relative level of gene expression of all analyzed transcripts was not affected
424   by the irrigation treatments ($P > 0.05$). In *A. alba*, all transcripts were present in the analyzed
425   samples. Relative gene expression of all but one transcript was not affected by the irrigation
426   treatments. We observed significant increase of relative gene expression of *TPCC* in response
427   to a decrease in water dose ($r = 0.8221$, $F = 20.8453$, $P = 0.001$).

428

429   **Discussion**
430   *Species-specific growth responses to irrigation and alterations in gene expression*

431   In this study of containerized-seedlings, large interspecific differences were observed in
432 the response of seedling growth to reduced irrigation. These differences were associated with
433 the classification of the different species. In agreement with our first hypothesis, the broadleaf
434 deciduous genera, *Fagus* and *Quercus,* exhibited a greater magnitude of response to reduced
435 irrigation than the two evergreen, conifer genera, *Abies* and *Pinus*. Within the deciduous species,
436 *F. sylvatica* was more greatly impacted by reduced water availability than *Q. petraea*. The
437 response of the deciduous species was in agreement with previous results obtained for seedlings
438 and older trees of *Q. petraea*, indicating that it should be classified as a drought-tolerant species
439 [70,71,72] and *F. sylvatica* as a drought-intolerant species [73,74]. Unexpectedly, the shade-
440 tolerant and climax species, *A. alba,* exhibited a weak phenotypic response to reduced level of
441 irrigation, but the enhanced expression of *TPCC* observed in needles may indicate greater levels
442 of stomatal closure in response to the lowest level of irrigation [58]. On the other hand, the
443 pioneer and shade-intolerant species, *P. sylvestris,* exhibited a positive growth response and
444 high morphological plasticity. The difference between the response of the studied conifers may
445 be the result of their contrasting growth phenology as seedlings. The slow-growing species, *A.*
446 *alba,* with its small amount of leaf biomass, requires less water than the faster growing, pioneer
447 species, *P. sylvestris.* Although all seedlings were exposed to drought during their rapid-growth
448 phase, growth in *Abies* is naturally terminated sooner than in *Pinus*; thus the ability of *Abies* to
449 respond to the decreased water availability may be restricted by its phenological cycle. This
450 conservative growth pattern of *A. alba* may represent a portion of a drought-avoidance strategy,
451 providing another example of a trade-off between survival and growth [75]. Seeds of *A. alba*
452 are also heavier than seeds of *P. sylvestris* and thus provide more resources to germinating
453 seedlings during their initial growth than seeds of *P. sylvestris*. This would provide an
454 advantage to *A. alba* seedlings, especially under water deficit conditions [76]. The interspecific
455 differences observed in our experiment resulted to some extent from a different species-specific
456 interception, which reduced amount of water reaching roots of seedlings growing in containers.
457 In mature *P. sylvestris* stands in Germany, mean precipitation interception was 32% [77], and
458 in leafed period in *F. sylvatica* forest in Belgium, the level of interception was 28% [78]. These
459 data cannot be directly compared with the interception of our study seedlings, but they allow to
460 estimate the importance of interception in the water balance of trees under natural conditions.
461 In our study, however, the interception effect on water amount reaching the substrate and roots
462 of seedlings in containers was substantially reduced: (1) Water was delivered at high pressure:
463 at the sprinklers the pressure was 4 bars, and at crowns of seedlings around 3.0 – 3.2 bars and

never lower than 2.5 bars. Due to high pressure, even after full leaf expansion, water reached the substrate in cavities; (2) We cultivated our plants from seeds, thus at the beginning of the experiment, the value of interception was 0 and during seeds' germination and initial growth, it did not substantially affect the amount of water irrigating the substrate. An effect of interception was important at the end of our experiment, after full leaf expansion. However, this effect of different interception might be at least partially compensated by the high pressure of irrigation water, high air humidity in the tunnel and foliar water absorption of seedlings which can be significant under water deficit [79].

The select genes allowed us to compare tolerance to reduced irrigation within each species. The relative abundance of most of the analyzed drought-response-related transcripts was not affected by the different levels of irrigation except for *ZAT11* in *Q. petraea* and *TPC1* in *A. alba* seedlings (Fig. 6). *ZAT11* was involved in oxidative stress-induced programmed cell death [80] (Qureshi et al. 2013) and positive regulation of primary root growth in *A. thaliana* [81]. Higher *ZAT11* could be associated with a higher production of reactive oxygen species due to drought stress. An increase in *TPC1* was involved in stomatal closure and abiotic stress responses in *Triticum aestivum* [82]. Although *TPC1* was proposed as a reference gene with stable expression for drought stress [58], we found the opposite result. In our study, higher abundance of this transcript can be related with a greater stomatal closure in *A. alba* under drought stress. Importantly, this suggests that the intraspecific selection may be initiated by regulated water deficit. Since only leaf mRNAs were analyzed in our study, it is plausible that the expression of these drought-response-related genes may have been higher in roots. Under a higher water deficit than that in our study, *Populus alba* × *Populus tremula* var. *glandulosa* increased transcript levels of several drought markers in leaves and roots [83]. In agreement with our results, Muilu-Mäkelä et al. [69] reported that the expression of genes involved in biosynthesis was not affected by drought stress in *P. sylvestris* seedlings. They did find, however, that the expression of the catabolism-related genes encoding diamine oxidase and polyamine oxidase were down-regulated in stems in response to drought. The increased level of *LEA* expression observed in that study in all parts of the seedlings indicates that the level of drought stress imposed on *P. sylvestris* seedlings in that study was higher than the level of drought stress imposed in the present study. A lack of significant expression or low expression of the selected stress related, potential marker genes in the studied species suggests that the water deficit stress was efficiently moderated by stomatal closure.

Seedling water status

Relative tissue water content was used as a measure of plant water status. In our study, organ-level water content decreases caused by water deficit were small especially in the conifers, suggesting an efficient control of transpirational water loss. This was especially true in *Abies*. In addition, seedlings' water status could be substantially improved by foliar absorption of intercepted water, especially under water deficit. In an earlier study, the substantial improvement of water status, exceeding 1.0 MPa water potential for drought-stressed *Juniperus monosperma* plants was observed, following precipitation on an experimental plot that excluded soil water infiltration [79].

Conifers at the same time showed much larger hydration of leaves across irrigation levels as compared to the two angiosperms. This may be attributed to the low amount of mechanical tissues and the large mesophyll content in juvenile needles. Interestingly, conifer root systems were much less hydrated than angiosperms, however since conifer roots constituted lower fraction of biomass than leaves, conifer seedlings held more water per gram total biomass than the angiosperm seedlings.

Our results suggest that the ability to accumulate and conserve foliar water may constitute a fundamental difference between drought-survival strategies of broadleaved deciduous and evergreen conifer species. This is supported e.g., by a tighter stomatal control, substantial decrease in sap flow rates and transpiration in *P. sylvestris* compared with more drought-tolerant *Quercus pubescens* [84,85]. Our suggestion is consistent with the higher growth rates of deciduous angiosperm seedlings achieved through a more intensive gas exchange, especially when expressed on the leaf mass basis. In the general scheme of ecological strategies, conifers appear to emphasize water conservation in contrast to specialization towards water acquisition and spending in seedlings of broadleaf angiosperms.

Biomass allocation

Evidence from individual and multi-species studies shows that plants typically respond to water deficit by increasing RMF and decreasing ABMR, LMF and SMF [15,21,7,75]. This pattern was most closely represented by the response of *P. sylvestris*. In contrast, we did not observe modification of RMF in any other species while, surprisingly, *Fagus* responded to water deficit by increasing LMF. While the lack of modification of biomass allocation in *Abies* is understandable given its lack of growth response, the unexpected increase in LMF with drought observed in *Fagus* may reflect the opposite trend of SMF. Such result may be explained

16

530   by the intrinsic influence of plant size on allocation ratios: small plants tend to have low fraction

531   of structural tissue (stem) and a large fraction of leaves; an effect often confounding the results

532   of experimental treatments or environmental factors [21].

533   Increased RMR as seen in droughted *Pinus,* is an important morphological adaptation

534   to low availability of underground resources [86,21]. Under prolonged drought, moderate

535   increases in root mass ratio may increase survival and ensure sufficient water supply for

536   photosynthesis, whereas increasing this ratio beyond a level required to ensure survival and

537   favourable water relations may impose construction and maintenance costs resulting in net

538   inhibition of plant growth. The role of biomass partitioning can be more important than either

539   gas exchange or phenology as a mechanism of drought response which has been suggested by

540   the results regarding *Brassica rapa* [75].

541   A decrease in growth under water deficit may be advantageous for plants if it leads to a

542   reduction of leaf transpiration area. However, under natural conditions, lower allocation to

543   leaves and overall plant size reduction reduce the odds for individual's success in competition

544   for light with other plants. This problem does not occur in container cultivation as long as

545   seedlings are spaced to prevent significant competition. Additionally, higher RMR of seedlings

546   acclimated to lower irrigation is an advantageous trait when they are planted in dry sites. Given

547   the prevalence of drought episodes, high seedling survival rates may be more important than

548   their initial growth rates and biomass production [52,87]. Thus, morphological acclimation of

549   seedlings to lower water doses in a nursery, especially stimulation of increase in RMF, may

550   benefit their survival in the planting sites. However, this expectation should be verified by

551   results of further experiments with seedlings of various species produced under the different

552   irrigation treatments in the container nursery and then planted and grown in natural conditions.

553

554   *Osmoregulation in response to regulated water deficit*

555   Plants synthesize saccharides, alcohols, and amino-acids including proline that

556   accumulate within the protoplast, especially in the vacuole, leading to a decrease in cell osmotic

557   potential [88,54,30]. Besides facilitating soil water extraction, some of these compounds,

558   including proline, perform protective roles against oxidative damage associated with reduced

559   gas exchange [89]. Although proline accumulates in response to water deficit in both

560   angiosperm trees [90] and conifers [91], in our experiment such increase in proline

561   concentration occurred only in angiosperm species but not in the conifers. In our two conifers

562   this result, together with the relative homeostasis of needle water content, suggests that needle-

563 level signal of water deficit was not sufficient to induce this biochemical response. Alternatively,

564 conifers might accumulate some alternative osmolytes [92]. The utility of proline as an indicator

565 of water deficit stress, although widely accepted [91], certainly depends on the species, ecotype

566 and stress intensity as well as tissue type [92,90].

567

568 *Leaf anatomy*

569 Modifications of anatomical leaf traits in response to reduced irrigation were rather

570 minor and involved small reduction in lamina thickness in *Quercus*, decrease in palisade cell

571 length in *Quercus* and *Fagus* and decreases in the amounts of xylem and phloem tissues as well

572 as in vessel diameters in these two angiosperms. In contrast, none of the conifer needles showed

573 significant modifications. The character of changes in the two broadleaved species was only

574 partly consistent with the previously described modifications induced by drought. Leaves that

575 express constitutive or induced xeromorphism are often thicker than mesomorphic leaves and

576 show greater development of palisade tissue [93,23,94]. Reduction, rather than increase, in leaf

577 thickness and palisade cell length observed in our study suggests occurrence of a leaf-level

578 water deficit during leaf expansion and may be classified as symptom of water stress rather than

579 adaptive modifications. On the other hand, the decrease in transverse areas of xylem and phloem

580 are consistent with acclimation to lower gas exchange. Such plastic changes have been reported

581 in both drought and salinity stressed plants [95,96]. The change in leaf conduit diameter have

582 rarely been examined in studies of drought acclimation, although the few studies reporting such

583 data also show that vessels or tracheids become narrower in leaves developing under drought

584 [97,98]. Such modification may reflect reduced demand for water transport capacity under

585 decreased stomatal conductance and, as an additional benefit, reduce the vulnerability of narrow

586 vessels to embolization by decreasing the probability of 'rare leaky pits' [99,100].

587 The quantitatively small responses of angiosperm leaves and lack of response of

588 gymnosperm needles to drought suggest that water deficit in meristematic and growing shoot

589 tissues at the time of leaf development was not severe enough to induce significant plasticity of

590 these traits (see Ivancich et al. [101] and Binks et al. [24] for similarly conservative responses).

591 It is likely that the adjustment of growth rate and/or allocation of biomass, coupled with

592 physiological adjustment, helped to at least partially offset the effect of lower availability of

593 water and preserve the plant water status at least during the period of leaf expansion. Moreover,

594 leaves of young seedlings are known to be anatomically less plastic than those of later

595 ontogenetic stages [102].

18

596

Conclusions

The divergent responses of tree species to regulated water deficit indicate that the irrigation regimes in container nurseries would benefit from species-specific adjustment according to water requirements. Our results indicate that in case of gymnosperm evergreen conifer species the quantity of irrigation water could be reduced by 25% (for *P. sylvestris*) to 75% (*A. alba*), and in case of angiosperm deciduous broadleaf (*F. sylvatica* and *Q. petraea*) by 25% relative to the full dose of 6 mm without significantly affecting growth and inducing morphological, biochemical and molecular mechanisms of defense against water deficit. Although in both angiosperms growth was sensitive to water deficit, only very modest induction of stress indicators was observed suggesting reduction of water dose could be considered when hardening to field conditions is required.

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Supplementary Material

Table 1. Target genes and efficiency of primers used during quantitative real-time PCR for the analysis of gene expression of leaves and needles.

Captions of Tables and Figures

Table 1. Effect of irrigation treatments (100%, 75%, 50% or 25 % of the recommended dose) on height (h), diameter at root collar (d) and h/d ratio in *Fagus sylvatica*, *Quercus petraea*, *Abies alba* and *Pinus sylvestris* seedlings. Means ± S.E. are given (n = 40). One-way analysis of variance *F*-values with degrees of freedom in the lower index for treatment effect and error term and the associated *P* values are shown separately for each species (***P<0.001, ** *P*<0.01, * *P*<0.05, n.s. – not significant). Shared letters in a column indicate that the pre-planned contrasts between means are not significant.

Fig. 1. Water contents (determined as percentage fresh mass) in leaves (LWC; a), roots (RWC, b) and stems (SWC; c) of *Fagus sylvatica*, *Quercus petraea*, *Abies alba* and *Pinus sylvestris*

952   seedlings grown at different irrigation treatments (100%, 75%, 50% and 25% of the

953   recommended dose). Bars indicate means ± SE (*n*=16). One-way analysis of variance *F*-values

954   for treatment effect and the associated *P* values are shown separately for each species (* *P*<0.05,

955   n.s. – not significant). Shared letters above bars indicate that the pre-planned contrasts between

956   means are not significant.

957   Fig. 2. Total plant dry mass of (a) *Fagus sylvatica* and *Quercus petraea*, and (b) *Abies alba* and

958   *Pinus sylvestris* seedlings grown at different irrigation treatments (100%, 75%, 50% and 25%

959   of the recommended dose). Bars indicate means ± SE (*n*=16). Note the different scales. One-

960   way analysis of variance *F*-values for treatment effect and the associated *P* values are shown

961   separately for each species (***P<0.001, n.s. – not significant). Shared letters above bars

962   indicate that the pre-planned contrasts between means are not significant.

963   Fig. 3. Patterns of biomass allocation in *Fagus sylvatica*, *Quercus petraea*, *Abies alba* and *Pinus*

964   *sylvestris* seedlings grown at different irrigation treatments (100%, 75%, 50% and 25% of the

965   recommended dose): aboveground to belowground mass ratio (ABMR; a), leaf mass fraction

966   (LMF; b), root mass fraction (RMF; c) and stem mas sfraction (SMF, d). Bars indicate means

967   ± SE (*n*=16). One-way analysis of variance *F*-values for treatment effect and the associated *P*

968   values are shown separately for each species (***P<0.001, ** *P*<0.01, * *P*<0.05, n.s. – not

969   significant). Shared letters above bars indicate that the pre-planned contrasts between means

970   are not significant.

971   Fig. 4. Leaf proline concentration of *Fagus sylvatica*, *Quercus petraea*, *Abies alba* and *Pinus*

972   *sylvestris* seedlings grown at different irrigation treatments (100%, 75%, 50% and 25% of the

973   recommended dose). Bars indicate means ± SE (*n*=16). Results of a two-way analysis of

974   variance with species and irrigation treatments as the fixed-factors are given (*F*-values for

975   treatment effect and the associated *P* values are shown). Shared letters above bars indicate that

976   the pre-planned contrasts between means are not significant. *P* symbols are: ***P<0.001, **

977   *P*<0.01, * *P*<0.05, n.s. – not significant.

978   Fig. 5. Anatomical parameters in leaves of *Fagus sylvatica* and *Quercus petraea*, and needles

979   of *Abies alba* and *Pinus sylvestris* leaves in seedlings grown under different levels of irrigation

980   (100%, 50% and 25% of the recommended levels). Bars represent the mean (±S.E.) (*n* = 8, *n* –

981   number of seedlings per species and irrigation treatment). Anatomical features such as leaf

982   thickness (a), palisade cell length (b), transverse midvein phloem (c) and xylem area (d), and

983   within-leaf mean (e) and maximal (f) conduit diameter are shown. Results of a two-way analysis

984   of variance with species and irrigation treatments as the fixed-factors are given (F-values for

985   treatment effect and the associated *P* values are shown). Shared letters above bars indicate that

986   the differences bewteen means was not significant. *P* symbols are: ***P<0.001, ** *P*<0.01, *

987   *P*<0.05, n.s. – not significant.

988   Fig. 6. Relative transcript abundance of select response-to-abiotic-stress genes in leaves of

989   *Fagus sylvatica*, *Quercus petraea*, and needles of *Abies alba* and *Pinus sylvestris* seedlings

990   grown under different levels of irrigation (100%, 75%, 50% and 25% of the recommended

991   level) (means±SE, n = 12, number of seedlings per species and irrigation treatment). One-way

992   analysis of variance F-values for treatment effect with degrees of freedom and the associated

993   *P* values are shown separately for each species (***P<0.001, ** *P*<0.01, * *P*<0.05, n.s. – not

994   significant). Shared letters above bars indicate that differences bewteen means were not

995   significant.

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1009   **Tables and figures**

1010   Table 1

Species	Irrigation treatment (% recommended dose)	h (mm)	d (mm)	h/d
Fagus sylvatica	25	104±5a	1.88±0.08a	57±2a
	50	124±5b	2.13±0.09b	59±2a
	75	185±7c	2.77±0.08c	68±3b
	100	225±11d	3.37±0.11d	67±3b
$F_{3, 156}$		62.5***	51.4***	6.0**
Quercus petraea	25	82±3a	1.90±0.07a	44±2a
	50	94±3b	1.97±0.06a	48±2ab
	75	117±7c	2.48±0.08b	48±3ab
	100	148±8d	2.78±0.08c	54±3b
$F_{3, 156}$		28.6***	35.8***	3.2*
Abies alba	25	35±1a	1.00±0.03a	36±1a
	50	37±1a	1.05±0.03a	36±1a
	75	38±1a	1.03±0.03a	38±1a
	100	38±1a	1.00±0.02a	38±1a
$F_{3, 156}$		n.s.	n.s.	n.s.
Pinus sylvestris	25	35±1a	1.01±0.03a	35±1a
	50	39±1b	1.06±0.03a	37±1a
	75	48±1c	1.17±0.04b	43±1b
	100	51±2c	1.18±0.04b	45±2b
$F_{3, 156}$		31.9***	5.4***	11.9***

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Fagus $F_{3,60}= 0.48$ *n.s.*
Quercus $F_{3,60}= 7.00$ ***
Abies $F_{3,60}= 0.60$ *n.s.*
Pinus $F_{3,60}= 7.28$ ***

Fagus $F_{3,60}= 3.93$ *
Quercus $F_{3,60}= 1.59$ *n.s.*
Abies $F_{3,60}= 0.60$ *n.s.*
Pinus $F_{3,60}= 1.39$ *n.s.*

Fagus $F_{3,60}= 0.90$ *n.s.*
Quercus $F_{3,60}= 3.79$ *
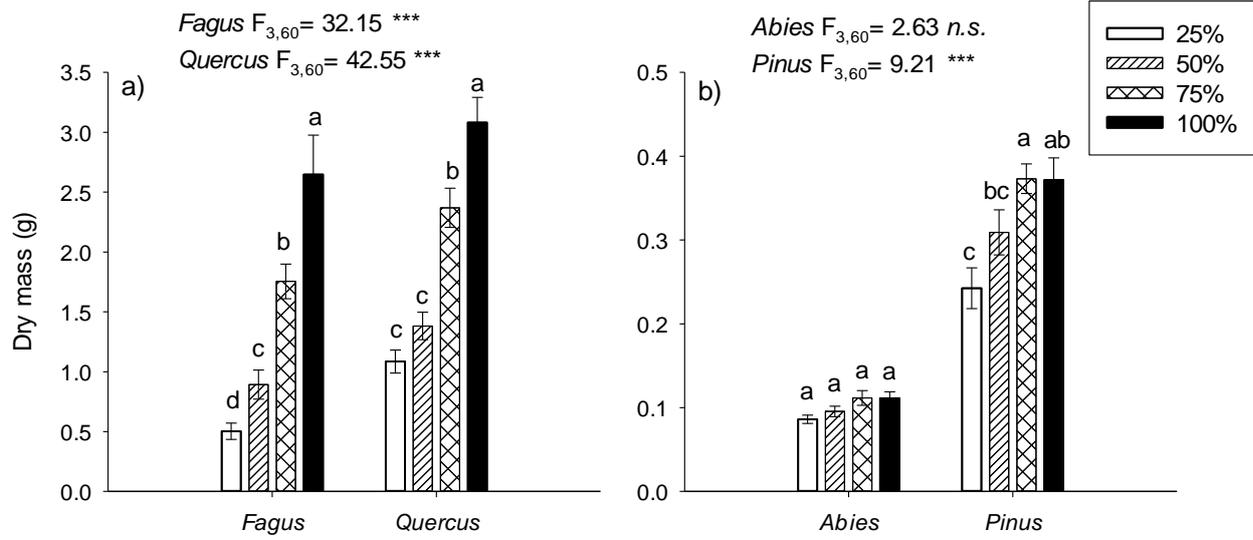*Abies* $F_{3,60}= 2.79$ *
Pinus $F_{3,60}= 1.40$ *n.s.*
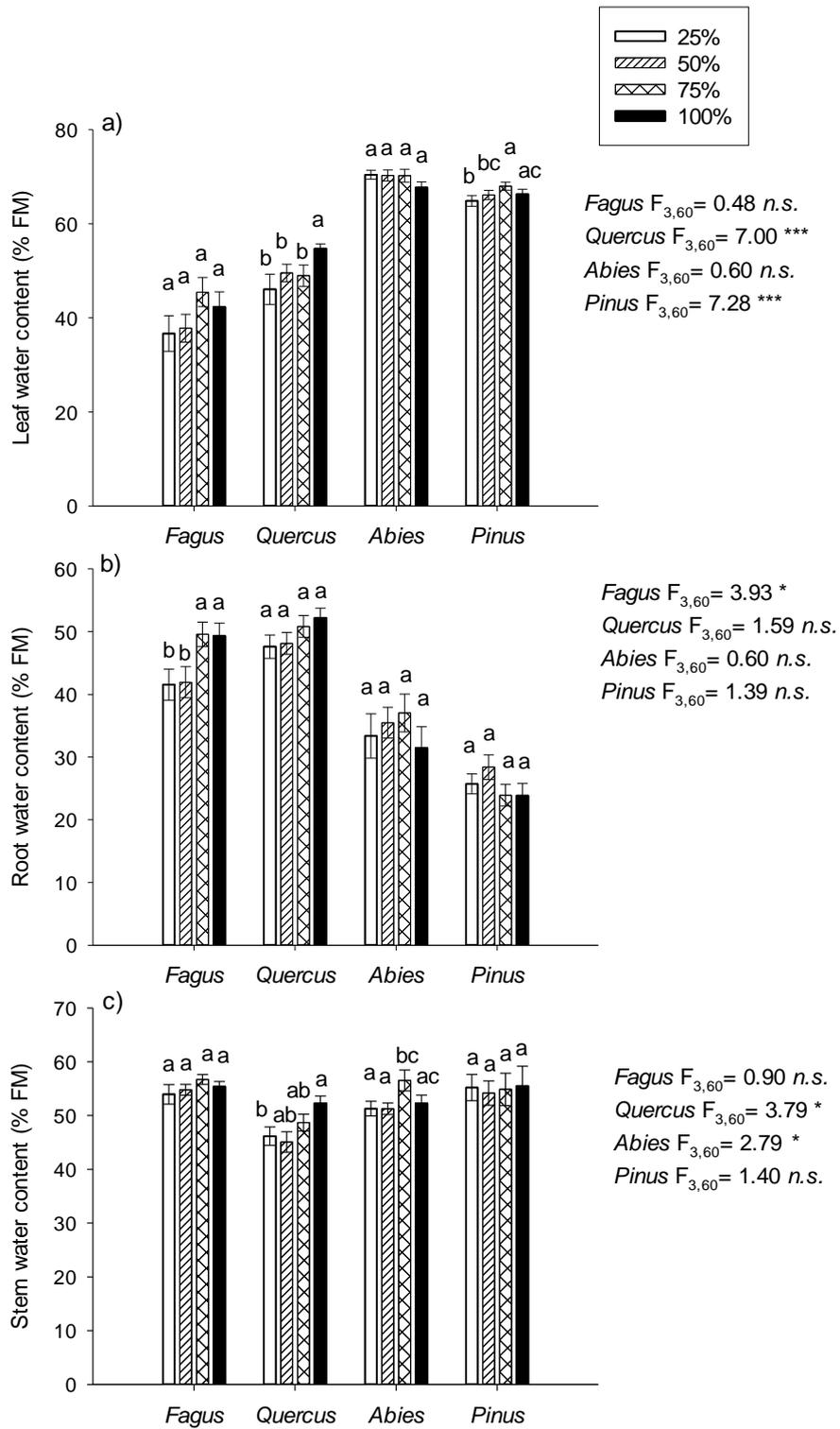
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1024   Figure 1

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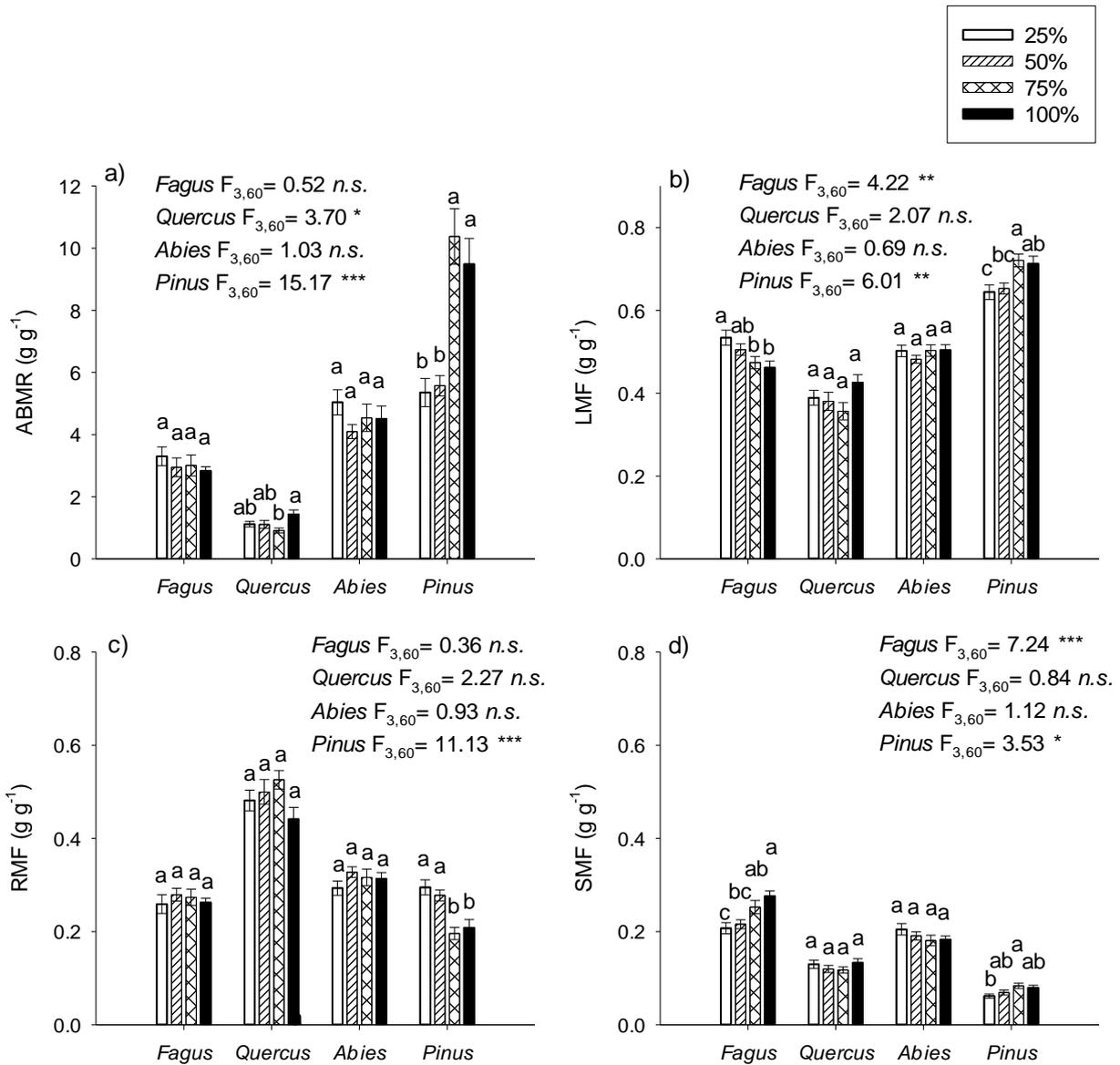
Figure 3

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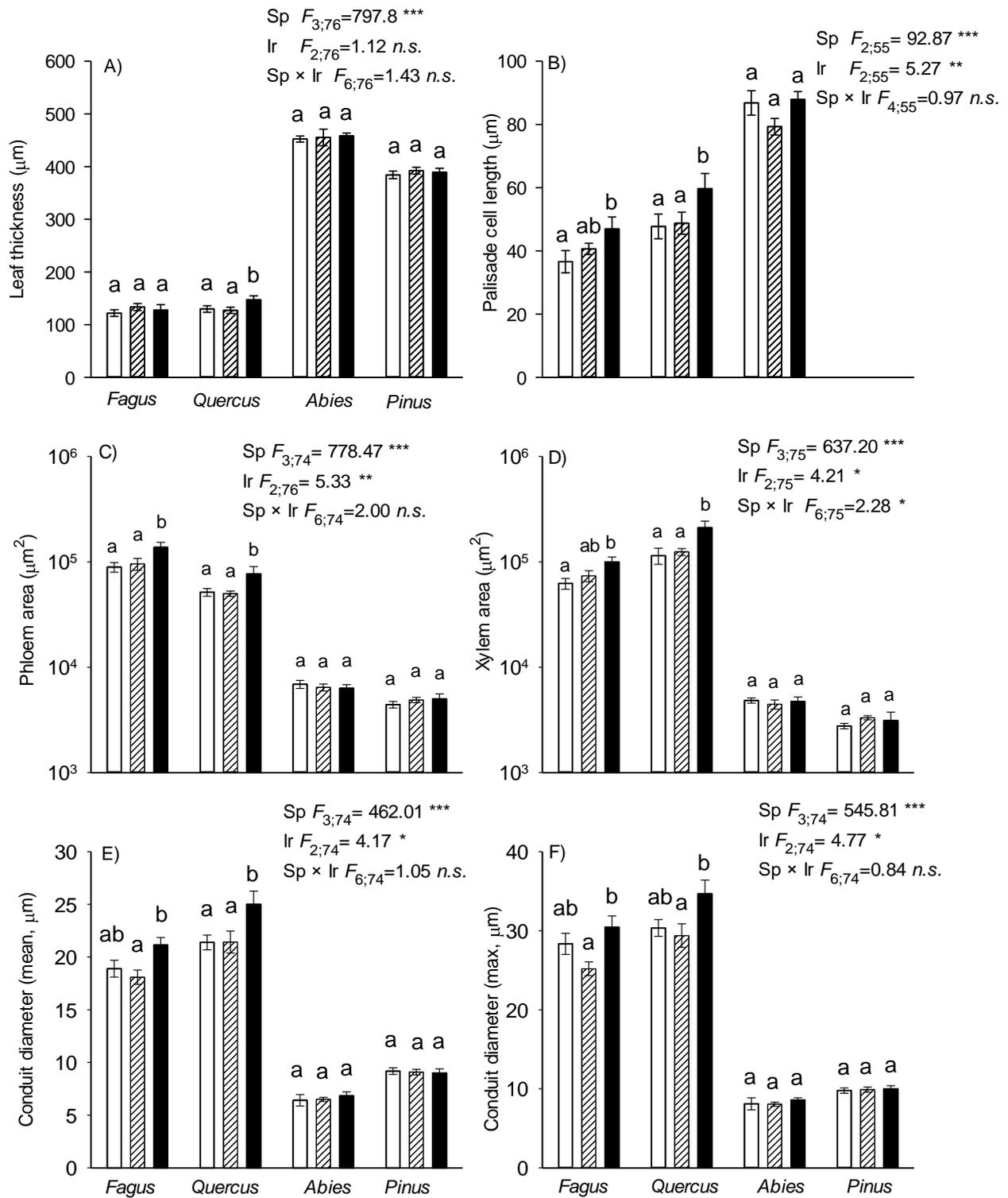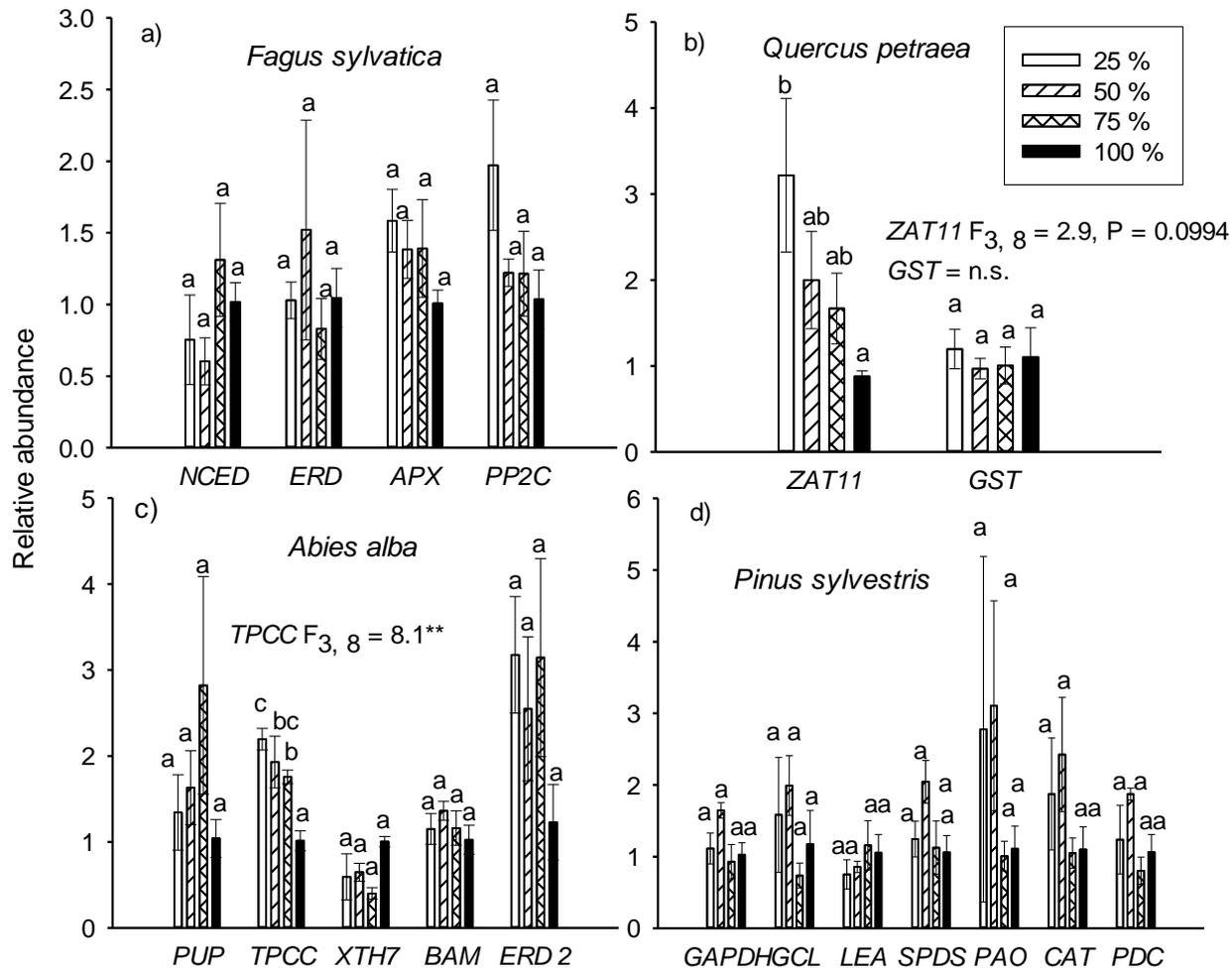1033

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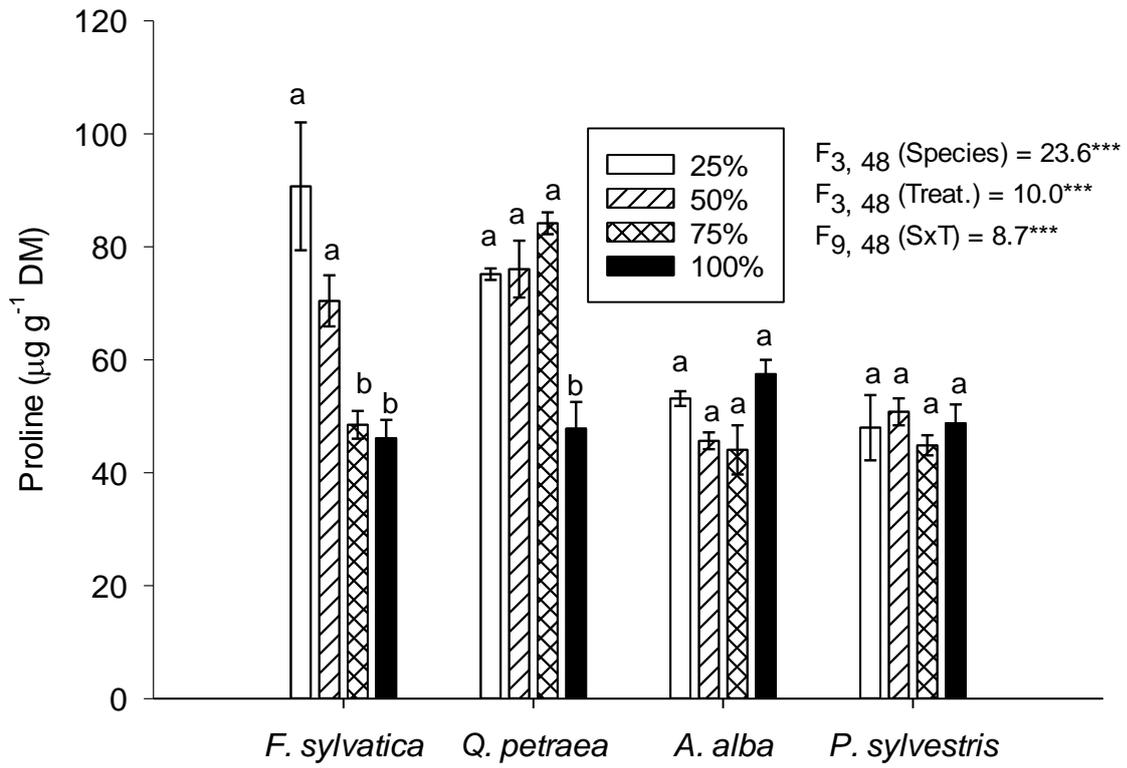
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