1    Research Papers

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3    **Title**

4    Morphological, biochemical and physiological traits of upper and lower canopy leaves

5    of European beech tend to converge with increasing altitude

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7    **Running Headline**

8    Upper and lower leaves converge with altitude

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22  **Summary**

23  The present work has explored for the first time acclimation of upper versus lower

24  canopy leaves along an altitudinal gradient. We tested the hypothesis that restrictive

25  climatic conditions associated with high altitudes reduce within-canopy variations of

26  leaf traits.

27  The investigated beech (*Fagus sylvatica* L.) forest is located on the southern slope of

28  the Hrubý Jeseník Mountains (Czech Republic). All measurements were taken on leaves

29  from upper and bottom parts of the canopy of mature trees (>85 years old) growing at

30  low (420 m a.s.l.), middle (720 m a.s.l.), and high (1100 m a.s.l.) altitudes.

31  Compared to trees at higher altitudes, those growing at low altitudes had lower stomatal

32  conductance, slightly lower $CO_2$ assimilation rate ($A_{max}$) and leaf mass per area (LMA),

33  and higher photochemical reflectance index, water use efficiency, and Rubisco content.

34  Given similar stand densities at all altitudes, the different growth conditions result in a

35  more open canopy and higher penetration of light into lower canopy with increasing

36  altitude. Even though strong vertical gradients in light intensity occurred across the

37  canopy at all altitudes, lower canopy leaves tended at high altitudes to acquire the same

38  morphological, biochemical and physiological traits as did upper leaves. While

39  elevation had no significant effect on nitrogen and carbon contents per unit leaf area,

40  LMA, or total content of chlorophylls and epidermal flavonoids in upper leaves, these

41  increased significantly in lower leaves at higher altitudes. The increases in N content of

42  lower leaves were coupled with similar changes in $A_{max}$. Moreover, high N content

43  coincided with high Rubisco concentrations in lower but not in upper canopy leaves.

44  Our results show that the limiting role of light in lower parts of the canopy is reduced at

45  high altitudes. A great capacity of trees to adjust the entire canopy is thus demonstrated.

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48 **Key-words:**

49 altitudinal gradient, $CO_2$ assimilation, flavonoids, light environment, leaf stoichiometry,

50 LMA, Rubisco

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**Introduction**

Climatic variation along altitudinal gradients provides an excellent and natural experimental set-up for investigating the possible impacts of climate change on terrestrial organisms and ecosystems (Körner 2007, DeFrenne et al. 2013). There are four primary atmospheric changes associated with altitude: decrease in partial pressure of gases, reduced temperature, reduced clear-sky turbidity, and higher fraction of ultraviolet radiation and precipitation. In contrast, wind velocity, soil conditions, and season length may not generally be related to altitude and may depend upon, among other things, slope orientation, topology and/or region (reviewed in Becker et al. 2007, Körner 2007).

In addition to studies on species distribution and composition of plant communities (Halbritter et al. 2013, Read et al. 2014), genomic divergence (Chapman et al. 2013), and interactions between host plant and herbivores or fungal pathogens (Hodkinson 2005), attention has also been given to the acclimation of morphological, biochemical and physiological traits of plants along an altitudinal gradient (e.g., Sakata et al. 2006, Kumar et al. 2008, Guerin et al. 2012). While these studies have focused mainly on herbaceous species and agricultural crops, possible differences in acclimation of leaves across the vertical profile of the forest canopy to growth conditions had not been studied.

Studies on deciduous forest and herbaceous species have shown an increase of leaf mass per area (LMA) and leaf nitrogen content per unit area with increasing altitude (Williams et al. 1995, Song et al. 2012). Other studies have reported increases in stomatal density, stomatal conductance, and light-saturated rate of $CO_2$ assimilation with increasing altitude (Hultine and Marshall 2000, Vats et al. 2009). Moreover, the maximum rates of Rubisco carboxylase activity and of photosynthetic electron transport

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have been shown to be higher for leaves from plants grown at high altitudes than for those grown at low altitudes (Fan et al. 2011), even as the activities of other enzymes associated with carbon assimilation have not shown significant differences with changing altitude (Kumar et al. 2008).

An exponential attenuation of solar radiation passing through a canopy leads to distinct light intensity across a vertical canopy profile. Leaves acclimate to their light environments by (i) modulation of leaf morphology, anatomy, and chloroplast ultrastructure (Boardman 1977, Lichtenthaler et al. 1981, Kubiske and Pregitzer 1997, Yano and Terashima 2001), and (ii) changes in their chemical composition, including in particular reallocation of nitrogen between photosynthetic components associated with light capture, thylakoid membrane composition, and $CO_2$ assimilation (Sims and Pearcy 1994, Eichelmann et al. 2005, Hikosaka 2005, Lichtenthaler et al. 2007). The thicker upper canopy leaves are characterized by lower water content, higher total chlorophyll and total carotenoid content per leaf area unit, as well as higher values for the Chl a/b ratio compared to the much thinner lower canopy leaves (Lichtenthaler et al. 2007). While upper leaves have higher rates of light-saturated $CO_2$ assimilation, which are associated with higher Rubisco content and stomatal conductance, lower leaves more effectively utilize low light intensities (Sims and Pearcy 1994, Urban et al. 2007). Lower canopy leaves play an important role in whole-canopy carbon fixation, particularly during cloudy days with prevailing diffuse radiation but also during hot sunny days when the stomatal conductance, $CO_2$ uptake and light-use efficiency of the uppermost sunlit leaves may be reduced (Urban et al. 2012a, Niinemets 2014a). It is not clear, however, how distinct growth conditions associated with different altitudes affect the vertical distribution and within-canopy variation of leaf traits.

101       Our main objective was to study the plasticity and possibly different acclimation

102    of upper and lower canopy leaves along the altitudinal gradient. To the best of our

103    knowledge, no comprehensive study had yet been undertaken on how the morphological,

104    biochemical and physiological traits of upper and lower canopy leaves are affected by

105    altitudinal gradient. Therefore, we aimed to investigate the within-canopy variations in

106    leaf structure (LMA), biochemistry (elemental stoichiometry; flavonoid, chlorophyll

107    and Rubisco content) and functioning ($CO_2$ assimilation rate, stomatal conductance,

108    photochemical reflectance index) of European beech (*Fagus sylvatica* L.) grown in a

109    forest with prevailing beech abundance at three different altitudes. The altitudinal

110    experiment was designed to test a hypothesis predicting that canopies respond to

111    changing climate by altered structure that may subsequently lead to reduced within-

112    canopy variations of morphological, biochemical and physiological leaf traits at high

113    altitudes. Since the asymmetrical acclimation of upper and lower canopy leaves has the

114    potential to cause a substantial change in the photosynthesis of forest canopies, this is a

115    key issue concerning altitudinal adaptations in plant ecophysiology.

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118    **Materials and methods**

119    *Site description*

120    The forest stand selected for this study is located on the southern slope of Mravenečník

121    Mountain (Hrubý Jeseník Mountains, 50°2' N, 17°9' E, Czech Republic). Leaf sampling

122    and physiological measurements were done on European beech (*Fagus sylvatica* L.)

123    trees naturally occurring at low (L; 420 m a.s.l.), middle (M; 720 m a.s.l.), and high (H;

124    1100 m a.s.l.) altitudes. As calculated from 30 years of data for L, M, and H altitudes,

125    respectively, the individual sites are characterized by gradients in mean annual air

126    temperature (7.59, 5.94, and 3.82 °C) and mean annual sum of precipitation (753, 891,

127    and 1083 mm). The mean monthly temperatures (2 m above the soil surface) and

128    monthly sums of precipitation during the investigated season (2013) are shown in Fig.

129    1. Both meteorological parameters were measured automatically in open areas close to

130    the investigated plots (up to 200 m distant).

131        Characteristics of the forest stands investigated are summarized in Table 1. A

132    stand with mature trees (>85 years old) was selected at each altitude. The stand

133    densities were 638, 772, and 763 trees ha$^{-1}$ at L, M, and H altitudes, respectively. L

134    trees had larger diameter at breast height and total tree height, basal area index, and leaf

135    area index as compared to M and H trees. Despite similar stand density, such a structure

136    of forest stands resulted in higher penetration of solar radiation at higher elevations as

137    compared to low ones (Table 1). Although long-term measurements of photosynthetic

138    photon flux (PPF) within the experimental stands could not be performed, PPF was

139    recorded using an LAI-2200 (Li-Cor, USA) with a quantum sensor (LI-190) above the

140    canopy and at the level of the investigated lower canopy leaves/branches. Data were

141    collected at maximum solar elevations (10:00–14:00 LMT). Upper canopy foliage was

142    exposed to a maximum PPF of 1500–2000 µmol m$^{-2}$ s$^{-1}$ irrespective of the elevation,

143    whereas lower canopy foliage received up to 150 (H), 105 (M), and 85 (L) µmol m$^{-2}$ s$^{-1}$

144    PPF on sunny days.

145        Two soil samples (0–20 cm depth) were taken at each of 13 trees (at a distance

146    ca 1 m to the west and to the east from the tree's base) at each altitude. Soil

147    characteristics were estimated on 2 mm fraction. Atomic-absorption spectroscopy was

148    used to assess Ca$^{2+}$, Mg$^{2+}$ and K$^+$ content. Content of P was determined

149    spectrophotometrically as a molybdate–phosphate complex and total N by distillation

150    after mineralization (Kjeldahl technique). Soil organic carbon was determined by

151    weight loss on ignition at 530 °C. Soil elemental concentrations and stoichiometry of

152    the plots investigated are summarized in Table 2.

153

154    *Physiological measurements and sampling procedures*

155    We evaluated biochemical and physiological parameters in leaves from the uppermost

156    and lowest canopy layers of the beech trees. Measurements were carried out on 13

157    representative trees from each altitude. Two leaves per tree and canopy layer with SSW

158    orientation were investigated. Branches with desired leaves were cut from the trees. The

159    cut end of each branch was immediately recut under water to remove xylem embolisms

160    and kept in water during the measurements. All branches were taken from healthy trees

161    showing no signs of damage.

162        Approximately 0.06 g of leaf fresh weight was sampled for analysis of Rubisco

163    (ribulose-1,5-bisphosphate carboxylase/oxygenase) enzyme content. After determining

164    the projection leaf area using a portable leaf area meter (Li-3000A, Li-Cor, USA), the

165    samples were immediately frozen in liquid nitrogen. Rubisco content was determined

166    by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a

167    Mini-PROTEAN 3 system (Bio-Rad, USA), as described by Urban et al. (2012b), using

168    purified Rubisco protein (Sigma-Aldrich) as a standard. The quantification of individual

169    bands was performed on an HP Scanjet 5590P running the program Advanced Image

170    Data Analyzer, version 3.23.001 (Raytest, Germany).

171        The elemental analyses of C and N were made using an automatic analyser

172    (Flash 2000, Thermo Scientific, USA). Leaf samples for elemental analyses (ca 100 mg)

173    were stored in liquid nitrogen after determination of projected leaf area. Before analysis,

174    each sample was dried to a constant mass in a drying oven (80 °C) for ca 2 days. The

175    leaf mass per area (LMA) ratio was defined as the ratio between leaf dry mass and

176     projected leaf area. Leaf moisture (Lm) in leaf samples was calculated as the ratio of

177     leaf fresh minus dry weight divided by leaf dry weight.

178        Light-saturated rates of $CO_2$ assimilation ($A_{max}$) and stomatal conductance

179     ($G_{Smax}$) were determined under ambient $CO_2$ concentration ($385 \pm 5$ µmol mol$^{-1}$) and

180     constant microclimatic conditions (leaf temperature: $25 \pm 1$ °C, relative air humidity:

181     $55 \pm 3\%$) using a Li-6400XT gas exchange system (Licor, Lincoln, NE, USA). Constant

182     saturating irradiance (1200 µmol m$^{-2}$ s$^{-1}$) was provided by LED light source of a LI-

183     6400-02B (Li-Cor, Lincoln, NE). In our previous studies (Lichtenthaler et al. 2007,

184     Urban et al. 2007) we had shown that such PPF is sufficient to saturate $CO_2$ assimilation

185     rate in upper canopy leaves of many tree species, while it has no photoinhibitory effects

186     on lower canopy leaves. The *in vivo* contents of epidermal flavonols and chlorophylls

187     (Chl a+b) were determined by Dualex 4 Flav (Force-A, Orsay, France). Leaf reflectance

188     spectra were measured in the wavelength range 350–2500 nm using a FieldSpec 4

189     HiRes spectroradiometer (ASD Inc., Boulder, CO, USA) coupled with leaf clip

190     reflectance probe (ASD Inc.). Three reflectance spectra per leaf were taken. The

191     photochemical reflectance index (PRI) was subsequently derived. This index expresses

192     an association with photosynthetic light use-efficiency and it is defined as the ratio of

193     reflectance (R) at 531 and 570 nm wavelengths: PRI = (R531 − R570) / (R531 + R570)

194     (Gamon et al. 1992, Peñuelas et al. 1995).

195        All measurements and samplings were done during the extended noon period

196     (10:00–14:00 LMT) and at two stages of growing season, the first characterized by

197     active growth (9–10 July) and the second by early senescence (16–17 September).

198

199     *Statistical analyses*

200 Before the analysis of variance, the normality of data for individual parameters was

201 tested using the Kolmogorov–Smirnov test for normality. A two-way fixed-effect

202 ANOVA model was used for the general analysis of altitude and leaf position effects

203 (see Supplementary Table 1).

204 To compare the data within graphs, a two-way ANOVA followed by a multiple

205 range test was performed to investigate the effects of altitude and leaf position within

206 canopy on biochemical, physiological, and morphological parameters. Tukey's post-hoc

207 ($P < 0.05$) test was used. All statistical tests were done using Statistica 12 (StatSoft,

208 Tulsa, USA).

209

210

211 **Results**

212 *Leaf structure and C:N stoichiometry*

213 The vertical position of a leaf within the canopy had a major effect on LMA and Lm

214 (Supplementary Table 1). Significantly lower LMA values were found in lower as

215 compared to upper canopy leaves. While the LMA values of upper leaves did not differ

216 with altitude, LMA values of lower leaves increased with rising altitude. This pattern

217 was similar for both measurement dates (Fig. 2a). Lm values were significantly higher

218 in lower than in upper canopy leaves at all altitudes and in both seasons. Lm of lower

219 leaves tended to decrease with altitude whereas Lm of upper leaves increased with

220 altitude (Fig. 2b).

221 Both nitrogen ($N_{area}$) and carbon ($C_{area}$) content per unit leaf area were closely

222 related to LMA ($R^2 = 0.926$ and 0.996 for $N_{area}$ and $C_{area}$, respectively; $P < 0.01$; data

223 not shown). The content of $N_{area}$ and $C_{area}$ as well as the C:N ratio were higher in upper

224 than lower canopy leaves in both sampling periods, but these markedly increased with

225  altitude in lower canopy leaves (Fig. 3a–d). Accordingly, differences in C:N ratio (Fig.

226  3e,f) between uppermost and bottom leaves tended to be smaller or to disappear at

227  higher altitudes (M and H) while large within-canopy differences were observed at low

228  altitude (L).

229

230  *Effect of altitude on $CO_2$ assimilation and stomatal conductance*

231  The altitudinal patterns of $A_{max}$ and $G_{Smax}$ changed during the season (Fig. 4a–d). While

232  in July the highest $A_{max}$ and $G_{Smax}$ values were achieved at the middle altitude (M), in

233  September both parameters gradually increased with rising altitudes. These differences

234  were not statistically significant, however. Upper canopy leaves showed higher $A_{max}$

235  compared to lower canopy leaves in both sampling periods and at all altitudes. The

236  $A_{max}/G_{Smax}$ ratio (Fig. 4e,f) – also referred to as intrinsic water use efficiency – was not

237  influenced by leaf position within the canopy, but it did show gradual decrease with

238  rising altitude.

239       The close relationships ($R^2 = 0.51$; $P < 0.01$; calculated for the whole dataset)

240  between $A_{max}$ and $G_{Smax}$ (Fig. 5) revealed that changes in $CO_2$ assimilation relate to

241  changes in stomatal conductance. Distinct relationships were found for upper and lower

242  canopy leaves, however, thus reflecting also the effect of leaf structure and biochemical

243  composition on $CO_2$ assimilation rate. Furthermore, a clear decrease in the asymptote of

244  this relationship, represented by the parameter *a* in the hyperbolic function applied (Fig.

245  5), was found at the end of summer.

246       Lower PRI values, associated with photosynthetic light-use efficiency, were

247  observed in upper as compared to lower canopy leaves all through the vegetation season

248  (Fig. 6). Particularly in September (Fig. 6b), PRI tended to decrease in both upper and

249  lower leaves with increasing altitudes.

250

*N partitioning and CO$_2$ assimilation*

Rubisco content per unit leaf area (Rubisco$_{area}$) was significantly higher in upper than

lower canopy leaves at all altitudes. Rubisco$_{area}$ tended to decrease with altitude in upper

leaves while slightly increasing in lower canopy leaves. These patterns were more

pronounced in July (Fig. 7; Supplementary Table 1). The relationship between N$_{area}$ and

Rubisco$_{area}$, analysed for the whole dataset, shows exponential growth (Fig. 8). However,

different relationships can be recognized for individual altitudes. Particularly at high

N$_{area}$ contents (N$_{area} \geq 2.0$ g m$^{-2}$), which are typical for upper canopy leaves, a lower

amount of nitrogen is allocated to Rubisco at high as compared to low altitudes. This is

reflected in the higher value of the *b* exponent in the exponential model applied (Fig. 8).

The relationship between *A*$_{max}$ and Rubisco$_{area}$ shows relatively high variation

(caused by *G*$_{Smax}$), and relationships for individual altitudes can be differentiated (Fig.

9). At the lowest altitude, the *A*$_{max}$ values reached the asymptotic level at relatively

lower concentrations of Rubisco as compared to the M and H altitudes (reflected by the

lower parameter *a* in the exponential model applied).

Total chlorophyll content estimated in upper and lower canopy leaves tended to

converge at high altitude in both periods (Fig. 10a,b). Generally, leaves from the canopy

bottom show lower content of epidermal flavonols than do the upper leaves (Fig. 10c,d).

Moreover, flavonol content determined by fluorescence technique tended to increase

with altitude in lower canopy leaves while no such differences were found in upper

leaves. A close link between carbon metabolism and the accumulation of flavonols has

been revealed by the relationship between carbon content in leaves and epidermal

flavonols (*R*$^2$ = 0.88–0.89; data not shown). This relationship showed the asymptotic

nature of flavonol accumulation at C$_{area}$ above 30 g m$^{-2}$. The relationship was slightly

275    shifted by time, with higher content of flavonols occurring in the later season. The

276    nitrogen balance index (NBI), calculated as the ratio of chlorophylls to flavonoids,

277    decreased with altitude in lower canopy leaves while remaining relatively constant in

278    upper leaves (Fig. 10e,f).

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280

281    **Discussion**

282    At similar tree density per hectare, the different growth conditions along the altitudinal

283    gradient resulted in a more open canopy at high altitudes. This was reflected in lower

284    LAI values and subsequently increased penetration of light into the canopy (Table 1).

285    Similarly, Lowman (1986) had reported that warm temperate forests have higher LAI as

286    compared to cold temperate forests and that this results in lower transmission of light

287    through the canopy (5.2% versus 7.5%). Canopy structure thus has a key effect on the

288    penetration of solar beams into lower canopy depths. At highest sun elevations, the

289    lower canopy leaves of H, M, and L trees investigated received up to 150, 105, and 85

290    $\mu$mol m$^{-2}$ s$^{-1}$ PPF, respectively, whereas the uppermost leaves were exposed to a

291    maximum PPF of 2000 $\mu$mol m$^{-2}$ s$^{-1}$ in clear sky conditions at all altitudes. The crowns

292    of all trees were thus considerably differentiated into a sunlit and a shaded part at all

293    altitudes investigated.

294    Our results show great capacity of *F. sylvatica* trees to adjust the morphological,

295    biochemical and physiological traits of the entire canopy. We found evidence

296    supporting the hypothesis that the climatic conditions along the altitudinal gradient

297    modulate the structure of forest canopies and thereby alter the local light environment.

298    In particular, the limiting role of low light intensities is pronounced under the

299    favourable climate conditions of low altitudes. A less limiting role of light was

300 meanwhile observed under climate-limiting conditions of high altitudes, where the

301 canopies achieve lower LAI values (Table 1). As discussed below, such asymmetrical

302 acclimation resulted in a convergence of morphological, biochemical and physiological

303 traits of upper and lower canopy leaves with increasing altitude.

304

305 *Leaf mass area (LMA) and leaf N stoichiometry*

306 It has been reported that LMA and $N_{area}$ increase with altitude in some functional groups

307 like forbs and angiosperm trees but do not vary in conifers (Williams et al. 1995, Read

308 et al. 2014). Our results for *F. sylvatica* show increasing LMA, $N_{area}$, and $C_{area}$ with

309 rising altitude in lower canopy leaves but not in upper leaves (Figs 2 and 3). To the best

310 of our knowledge, such an asymmetrical response has not previously been reported.

311     LMA is significantly modulated by, among other factors, light intensity,

312 temperature and nutrient availability (Poorter et al. 2009), i.e., by variables that decrease

313 with canopy depth and altitude. Higher transmittance of light into lower canopy depths

314 at high altitudes (Table 1) is likely the most important reason for increased LMA of

315 lower canopy leaves. In addition, however, developmental constraints on high-elevation

316 plants may decouple leaf N content from soil N content due to restricted root activity at

317 low temperatures (Pregitzer et al. 2000) or the dilution of N and other nutrients in leaf

318 tissues may be inhibited due to restricted growth (Körner 1989, 2007). These

319 hypotheses are supported, respectively, by the relatively low differences of $N_{area}$ values

320 in leaves (Fig. 3) which are in contrast to the large differences in total N content in soil

321 across the altitudinal gradient (Table 2) or by higher $N_{area}$ values in lower canopy *F.*

322 *sylvatica* leaves at high elevations (Fig. 3). Finally, the decrease in temperature with

323 higher altitudes may additionally contribute to an increase in LMA of lower canopy

324 leaves. For example, Atkin et al. (2006) had noted that lowland *Plantago* species grown

325     at low temperatures increased LMA. This was associated with increased photosynthetic

326     capacity, thus demonstrating cold acclimation of lowland species.

327

328     *Accumulation of chlorophylls and flavonols*

329     We found a decrease in total chlorophyll content in upper canopy leaves at the highest

330     altitude studied, which is in accordance with the literature (Roblek et al. 2008, Prakash

331     et al. 2011). The total chlorophyll content in lower canopy leaves presented the opposite

332     trend, however, as it increased with altitude. Accordingly, upper and lower canopy

333     leaves at the highest altitude had approximately the same amount of chlorophylls (Fig.

334     10). Evans and Poorter (2006) had found that changes in LMA and nitrogen partitioning

335     between proteins and photosynthetic pigments within leaves are closely coupled in the

336     process of light acclimation. Plants grown in low-light conditions partitioned a larger

337     fraction of leaf nitrogen into light-harvesting proteins and proteins associated with

338     effective photochemical reactions on thylakoid membrane (Boardman 1977, Seemann et

339     al. 1987, Sims and Pearcy 1994). In contrast to lower canopy leaves, upper leaves invest

340     nitrogen primarily into photosynthetic enzymes and hence have greater demand for

341     carbon dioxide per unit area (Körner and Diemer 1987).

342        In addition, we observed a significant increase in flavonol content in lower

343     canopy leaves along the altitudinal gradient, whereas no differences between altitudes

344     were observed in upper canopy leaves (Fig. 10). We found strong relationships between

345     $C_{area}$ (Fig. 3c,d) and accumulation of epidermal flavonols, which is in accordance with

346     the previous finding that the biosynthesis of flavonoids, particularly phenylpropanoid-

347     derived compounds, is closely related to carbon–nutrient balance (Koricheva et al. 1998,

348     Peñuelas and Estiarte 1998). The synthesis of carbon-based secondary metabolites is

349     further determined by specific demands (e.g., osmolytes under drought stress,

350  antioxidants under ozone stress) induced by an unfavourable growth environment. The

351  synthesis of flavonoids, tannins, and hydroxycinnamate esters, among other metabolites,

352  may thus represent an alternative pathway for the dissipation of excessive radiation

353  energy and consequently may contribute to enhanced antioxidant capacity of the cell

354  (Grace and Logan 2000), particularly under the stress conditions of high elevations.

355

356  *Changes in Rubisco content*

357  Rubisco content per unit leaf area in upper and lower canopy leaves tended to converge

358  at higher altitudes (Fig. 7), particularly due to reduced Rubisco content in upper canopy

359  leaves. This is consistent with a gradually decreased allocation of N to Rubisco in upper

360  canopy leaves with increasing altitude (Fig. 8). It is consistent, too, with previous

361  findings that cold acclimation of plants, including induction of antifreeze proteins and

362  changes in the membrane composition (Janda et al. 2007), represent an important sink

363  of nitrogen.

364      Although the paradigm of N-based photosynthetic machinery assumes that N-

365  containing enzymes are fully active, several studies have shown that Rubisco may not

366  be fully active in naturally growing leaves (Eichelmann et al. 2005, Urban et al. 2012b).

367  The relatively low $A_{max}$ at high Rubisco contents observed in trees at low altitudes

368  indicates the Rubisco to be in enzymatically inactive forms. This may imply that

369  inactive Rubisco serves as nitrogen storage, especially in upper canopy leaves of trees

370  growing at low altitudes (Fig. 9). Similarly, Sakata et al. (2006) had reported an

371  impairment of Rubisco content and its activity in upper canopy leaves of *Aconogonum*

372  *weyrichii* along an altitudinal gradient as well as during the vegetation season.

373      The activity of photosynthetic enzymes – in contrast to light absorption – is

374  reduced at low temperatures and thus leads to an increased risk of photo-oxidative

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375   damage (Tsonev and Hikosaka 2003). Therefore, plants adapt to the low growth

376   temperatures associated with high altitudes by increasing the Chl a/b and Rubisco/Chls

377   ratios (Strand et al. 1999), reallocating nitrogen to fructose-1,6-phosphatase (Hikosaka

378   2005) or to antifreeze proteins (Yeh et al. 2000), raising the de-epoxidation state of

379   xanthophyll pigments (Molina-Montenegro et al. 2012), and/or accumulating UV-

380   screening pigments in the epidermis (Koricheva et al. 1998, Filella and Peñuelas 1999,

381   Roblek et al. 2008). These mechanisms thus document a strong modulatory effect of

382   growth temperature on plant/leaf acclimation to a local radiation regime.

383

384   *$CO_2$ assimilation rate and photochemical efficiency*

385   The aforementioned changes in morphological and biochemical traits of leaves

386   consequently result in a convergence of physiological functions of upper and lower

387   canopy leaves at higher altitudes, and in particular of assimilation capacity ($A_{max}$; Fig.

388   4a,b) and light-use efficiency as measured by the proxy PRI (Fig. 6). The rate of $CO_2$

389   uptake, however, was significantly controlled by stomatal conductance (Fig. 5).

390   Stomatal density, and presumably stomatal conductance, generally increases with

391   altitude (Körner and Cochrane 1985, Vats et al. 2009), but this was confirmed for both

392   upper and lower canopy leaves only at the end of the vegetation season (Fig. 4c,d). In

393   July, the hottest and driest period, the highest stomatal conductance was found for

394   altitude M, likely the site with the greatest local water availability.

395   As PRI has been associated with photosynthetic light use-efficiency (Gamon et

396   al. 1992, Peñuelas et al. 1995), our results suggest a trend, at least in September,

397   towards lower light-use efficiency at high altitudes (Fig. 6). This phenomenon has been

398   observed previously for *Quercus ilex* in areas of the Iberian Peninsula (Filella and

399   Peñuelas 1999).

400        In our previous studies (Lichtenthaler et al. 2007, Urban et al. 2007), we have

401    shown that leaves of *F. sylvatica* respond to insufficient light conditions primarily by

402    reduced LMA, which leads to significantly higher $A_{max}$ per leaf weight unit in lower

403    canopy leaves of *F. sylvatica* as compared to upper canopy leaves. Similarly, little

404    variation in the mass-based traits LMA, $N_{area}$, and $A_{max}$ was found in a study of *Quercus*

405    *ilex* by Niinemets (2014b). Such acclimation to low light intensities is regarded as a

406    typical response of shade-intolerant species (Kubiske and Pregitzer 1997). Nevertheless,

407    restricted growth conditions associated with high altitudes have a potential to stimulate

408    biochemical acclimation (represented for example by changes in $N_{area}$, chlorophyll and

409    Rubisco contents) in lower canopy leaves of *F. sylvatica*.

410

411

412    **Conclusion**

413    Generally, with increasing altitude lower canopy leaves tended to acquire the same traits

414    as upper canopy leaves. Nevertheless, there were strong vertical gradients in light

415    intensity across a canopy at all altitudes investigated. Under similar stand density,

416    restrictive growth conditions result in a more open canopy and higher penetration of

417    light into lower canopy with increasing altitude. An asymmetrical acclimation of upper

418    and lower canopy leaves thus resulted in a convergence of their morphological (LMA),

419    biochemical ($N_{area}$, $C_{area}$, Chls, Flavs, $Rubisco_{area}$), and physiological ($A_{max}$, $G_{Smax}$, PRI)

420    traits with increasing altitude. The beech forest responded mainly by changing the traits

421    of lower canopy leaves along the elevation gradient and thus showed a great capacity

422    for the tree to adjust its entire canopy to cope with changing conditions. Such plasticity

423    in the acclimation of leaves has the potential to cause a substantial change in the

424 photosynthesis of forest canopies and in their contribution to the overall carbon balance

425 of vegetation.

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589

590 **Table 1.** Tree age and mean values *(standard deviations)* of total tree height (Height),

591 stem diameter at breast height (DBH), basal area index (BAI), and leaf area index (LAI)

592 of European beech (*Fagus sylvatica*) trees growing at low (L; 400 m a.s.l.), middle (M;

593 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Transmittance (Tr) of photosynthetic

594 photon flux (PPF) was calculated as the ratio of PPFs above the canopy to those at the

595 level of lower canopy leaves/branches investigated at maximum solar elevations

596 (10:00–14:00 LMT) and clear sky conditions. Different letters denote significantly

597 different values at $P < 0.05$ ($n = 13$). BAI and LAI were estimated using an LAI-2200

598 optical plant canopy analyser (Li-Cor, USA) and represent the area of branches and

599 main stems and the total area of leaves per m$^2$ of land, respectively. Different

600 superscript letters denote significantly different values at $P < 0.05$ ($n = 13$).

601

| Altitude | Age | Height | DBH | BAI | LAI | Tr |
|---|---|---|---|---|---|---|
| | years | m | m | m$^2$ m$^{-2}$ | m$^2$ m$^{-2}$ | % |
| L | 95+ | 27[a] *(3.0)* | 0.51[a] *(0.09)* | 2.3[a] *(0.2)* | 12.5 *(1.03)* | 4.3 |
| M | 85+ | 19[b] *(3.8)* | 0.33[b] *(0.09)* | 1.4[b] *(0.2)* | 11.4 *(1.69)* | 6.3 |
| H | 100+ | 21[b] *(1.8)* | 0.37[b] *(0.04)* | 1.1[b] *(0.1)* | 7.2 *(0.04)* | 8.4 |

602

603

604 **Table 2.** Mean values *(minimum–maximum values)* of organic carbon (Corg), total

605 nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg), and potassium (K)

606 content in the soils of three experimental plots located at low (L), middle (M), and high

607 (H) altitudes. Different superscript letters denote significantly different values at $P <$

608 0.05 ($n = 13$).

| Altitude | Corg | N | P | Ca | Mg | K | C:N | C:P | N:P |
|---|---|---|---|---|---|---|---|---|---|
| | g kg$^{-1}$ | g kg$^{-1}$ | mg kg$^{-1}$ | mg kg$^{-1}$ | mg kg$^{-1}$ | mg kg$^{-1}$ | g g$^{-1}$ | g mg$^{-1}$ | g mg$^{-1}$ |
| L | 35$^a$ *(27-53)* | 1.5$^a$ *(0.9-2.3)* | 3.96$^b$ *(0.55-8.50)* | 715$^b$ *(220-1079)* | 80$^b$ *(50-106)* | 73$^a$ *(48-98)* | 24.0 | 8.9 | 0.4 |
| M | 75$^b$ *(59-99)* | 3.8$^b$ *(2.7-5.7)* | 0.85$^a$ *(0.00-5.05)* | 799$^b$ *(381-1383)* | 75$^b$ *(47-126)* | 120$^b$ *(71-188)* | 20.0 | 87.6 | 4.5 |
| H | 109$^c$ *(65-152)* | 4.7$^c$ *(2.2-6.1)* | 1.84$^a$ *(0.00-6.05)* | 336$^a$ *(258-497)* | 49$^a$ *(30-73)* | 142$^b$ *(85-208)* | 23.1 | 59.2 | 2.6 |

609

610

**Figure Legend**

**Fig. 1.** Annual courses of monthly mean air temperature (2 m above the ground) and monthly sums of precipitation measured on an open area close to the investigated plots in 2013. The plots are located along the altitudinal gradient: low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.).

**Fig. 2.** Leaf mass per area ratio (LMA; **a**, **b**) and leaf moisture (Lm; **c**, **d**) in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars show standard deviations ($n = 13$ trees). Identical superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$).

**Fig. 3.** Elemental analyses of total nitrogen ($N_{area}$; **a**, **b**) and carbon ($C_{area}$; **c**, **d**) contents per unit leaf area in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars show standard deviations ($n = 13$ trees). Identical superscript letters indicate homogeneous groups with statistically non-significant differences ($P > 0.05$).

**Fig. 4.** Light-saturated (1200 µmol m$^{-2}$ s$^{-1}$) rate of $CO_2$ assimilation ($A_{max}$; **a**, **b**), stomatal conductance ($G_{Smax}$; **c**, **d**), and intrinsic water use efficiency ($A_{max}/G_{Smax}$; **e, f**) in upper canopy (open columns) and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns represent means, and error bars

636  show standard deviations ($n = 13$ trees). Identical superscript letters indicate

637  homogeneous groups with statistically non-significant differences ($P > 0.05$).

638

639  **Fig. 5.** Relationship between light-saturated rate of $CO_2$ assimilation ($A_{max}$) and

640  stomatal conductance ($G_{Smax}$) in upper canopy (open circles) and lower canopy (opaque

641  circles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),

642  middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The hyperbolic functions

643  ($y = a / (1 + \exp(- (x - x0) / b))$) were fitted separately for upper (a = 16.35, b = 0.073,

644  x0 = 0.083, $R^2 = 0.89$, $P < 0.01$ in July and a = 10.36, b = 0.035, x0 = 0.075, $R^2 = 0.74$,

645  $P < 0.01$ in September) and lower canopy leaves (a = 11.08, b = 0.037, x0 = 0.068, $R^2 =$

646  0.66, $P < 0.01$ in July and a = 9.37, b = 0.062, x0 = 0.095, $R^2 = 0.82$, $P < 0.01$ in

647  September).

648

649  **Fig. 6.** Photochemical reflectance index (PRI) estimated on the basis of full reflectance

650  spectra (350–2500 nm) in upper canopy (open columns) and lower canopy (opaque

651  columns) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),

652  middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. Columns = means, error

653  bars = standard deviations, $n = 13$ (trees). Identical superscript letters indicate

654  homogeneous groups with statistically non-significant differences ($P > 0.05$).

655

656  **Fig. 7.** Total content of Rubisco enzyme (ribulose-1,5-bisphosphate

657  carboxylase/oxygenase) per unit leaf area in upper canopy (open columns) and lower

658  canopy (opaque columns) leaves of European beech (*Fagus sylvatica*) growing at low

659  (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes.

660  Columns = means, error bars = standard deviations, $n = 13$ (trees). Identical superscript

661 letters indicate homogeneous groups with statistically non-significant differences ($P >$

662 0.05).

663

664 **Fig. 8.** Relationship between total Rubisco content per unit leaf area and total nitrogen

665 content per unit leaf area ($N_{area}$) in upper canopy (u.c.; circles) and lower canopy (l.c.;

666 triangles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),

667 middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The exponential function

668 y = a * exp(b * x) was fitted to the data irrespective of leaf position within a canopy and

669 time of season for low (a = 1.18, b = 0.996, $R^2$ = 0.88; $P <$ 0.01), middle (a = 1.92, b =

670 0.676, $R^2$ = 0.67, $P <$ 0.01), and high (a = 1.40, b = 0.743, $R^2$ = 0.60, $P <$ 0.05) altitudes.

671

672 **Fig. 9.** Relationship between light-saturated rate of $CO_2$ assimilation ($A_{max}$) and total

673 Rubisco content per unit leaf area in upper canopy (u.c.; circles) and lower canopy (l.c.;

674 triangles) leaves of European beech (*Fagus sylvatica*) growing at low (L; 400 m a.s.l.),

675 middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.) altitudes. The exponential rise to

676 maximum function (y = a * (1 − exp(−b * x))) was fitted to the data irrespective of leaf

677 position within a canopy and time of season for low (a = 7.75, b = 0.362, $R^2$ = 0.40, $P <$

678 0.01), middle (a = 19.04, b = 0.096, $R^2$ = 0.59, $P <$ 0.01), and high (a = 10.18, b = 0.326,

679 $R^2$ = 0.26, $P <$ 0.01) altitudes.

680

681 **Fig. 10.** Total chlorophyll (a+b) content (Chls; **a**, **b**), epidermal content of flavonols

682 (Flavs; **c**, **d**), and nitrogen balance index (NBI; **e**, **f**) in upper canopy (open columns)

683 and lower canopy (opaque columns) leaves of European beech (*Fagus sylvatica*)

684 growing at low (L; 400 m a.s.l.), middle (M; 750 m a.s.l.), and high (H; 1100 m a.s.l.)

685 altitudes. Columns = means, error bars = standard deviations, $n$ = 13 (trees). Identical

686    superscript letters indicate homogeneous groups with statistically non-significant

687    differences ($P > 0.05$). Chlorophyll content was estimated on the basis of differential

688    transmission for two near-infrared wavelengths. Epidermal content of flavonols was

689    estimated based on the ratio of chlorophyll fluorescence induced by UV and red light.

690    NBI was determined as the Chls/Flavs ratio.