

Biochemical acclimation of European beech (*Fagus sylvatica* L.) saplings to canopy opening

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Abstract

The biochemical acclimation of European beech (*Fagus sylvatica* L.) saplings to canopy openings caused by various forest cuttings or natural disturbances was assessed in the 'Petrohan' training and experimental forest range in the Western Stara Planina Mountains. We examined changes in the activity of key enzymatic antioxidants, the accumulation of reducing sugars, the synthesis of phenols and flavonoids, and the content of photosynthetic pigments. Our findings indicated that among the antioxidant enzymes studied, catalase showed the most significant increase in activity in response to the canopy openings. The increase in superoxide dismutase activity was less pronounced, while guaiacol peroxidase was only activated when the saplings were fully exposed to sunlight after the removal of shade from older trees. More pronounced defensive response was observed in the accumulation of reducing sugars and synthesis of phenolics, particularly at higher altitudes and in areas with larger canopy openings. Illumination emerged as primary factor influencing flavonoid accumulation. Following canopy openings, European beech leaves often exhibited reduced chlorophyll content, particularly chlorophyll *b*, in favour of synthesizing more carotenoids. Cutting all mature trees during the group selection harvest practice induced stress on saplings similar to that observed after a large disturbance. Further studies on the influence of orography on patch location could enhance understanding of the mechanistic control of illumination and the stress experienced by saplings of shade-tolerant species like European beech.

Key words: catalase, guaiacol peroxidase, flavonoids, phenols, photosynthetic pigments, superoxide dismutase.

Introduction

European beech (*Fagus sylvatica* L.) is one of the most widely spread tree species in European forests, and it reaches its southeastern natural spread limit in the

Bulgarian mountains. In these borderline areas, populations of European beech are often subjected to stress due to the variation of one or more environmental factors that approach the species' biological tolerance limit. Forest cuttings or distur-

bances can substantially alter the light environment experienced by forest saplings (Kozłowski 2002, Dietze and Clark 2008). The extent of this change is influenced by factors, such as type and intensity of cutting or disturbance, spatial distribution of canopy openings, and initial forest structure. Different cutting practices can create spatial heterogeneity in resource availability, such as light, water, and CO₂. For example, forest cuttings often produce edges between cleared areas and the remaining forest. These edge effects can increase light penetration under the canopy, creating transitional zones with higher light levels compared to the forest interior (Matlack and Litvaitis 1999, Zheng and Chen 2000). In selective logging or partial cutting scenarios, some trees remain in the stand, providing partial shade and creating a dappled light environment with varying shade and sun flecks, which can stimulate regeneration, sapling physiology, and growth (Wagner et al. 2010, 2011). On the other hand, significant canopy openings after clear-cutting or windthrow lead to a drastically altered environment that can be particularly stressful for saplings (Oliver 1980), especially for late-successional species like European beech. Large canopy openings expose beech saplings to heightened environmental stresses, including elevated temperatures and greater fluctuations in soil moisture. This altered environment poses a potential threat to sapling growth and survival.

Few studies have explored the influence of different silvicultural systems or natural disturbances on biochemical acclimation in forests, which is the earliest and most critical stage of sapling adjustment to changing environmental factors. Canopy openings, whether caused by natural disturbances or silvicultural practices, further increase stress, especially on under-

storey saplings. The stress resulting from the stand's opening affects individual saplings unevenly, giving a haphazard character to the litter during this early stage of beech forest development. Acclimation of shade-developed leaves or saplings to increased light may depend on their ability to minimize the magnitude or duration of photoinhibition while enhancing photosynthetic capacity (Naidu and DeLucia 1998). The reactions of individual saplings are initially expressed at the biochemical and physiological levels—manifesting in changes in fundamental indicators, such as antioxidant defence activity, photosynthesis, respiration, and transpiration. These modifications lead to changes in the carbon and water balance of the saplings, potentially triggering gradual exhaustion (Lamloom et al. 2016). Schulze et al. (2019) note that plants rarely experience optimal levels of all essential abiotic factors. Stress responses, in turn, can have adverse effects on growth and other plant processes due to compromises in carbon balance. Under environmental pressure, plants allocate resources to their defence systems, which involve various biochemical, physiological, anatomical, and morphological mechanisms to adapt to their conditions. According to Naidu and DeLucia (1998), the ability to acclimate existing leaves to changing environmental conditions may enable forest tree saplings to exploit canopy gaps when conditions are unfavourable for new leaf production.

Antioxidant systems have been frequently studied in tree species with respect to drought or temperature compensation (Polle and Rennenberg 1994, Rennenberg et al. 2006). As Foyer et al. (1994) pointed out, high levels of incident sunlight combined with extreme temperatures can lead to photoinhibition and oxidative stress in photosynthetically

active tissues due to imbalance between light capture and light utilization for assimilation. Several authors have noted that heat and drought stress increase the production of reactive oxygen species (ROS) (Todorova et al. 2022, 2023, 2024; Carvalho and Helena 2008; Lee et al. 2009; Impa et al. 2012). Biochemical acclimation begins with the plant's need to neutralize these oxygen radicals, which are produced under conditions that deviate significantly from the species' optimal environment. Even during the storage of beech seeds, accumulation of free ROS, such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), is observed, and their levels correlate with a loss of seed viability (Pukacka and Ratajczak 2005). However, the most significant challenges occur in the survival of seedlings and saplings. Polle et al. (2001) observed increased oxidative stress as European beech saplings' leaves aged.

Different enzymatic mechanisms for antioxidant protection under stressed conditions are well known. Kraj (2016) pointed out that, except for catalase (CAT), the activity of antioxidative enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase is highest during the pre-senescence stage of leaves. Popović et al. (2015) noted that the combined analysis of the specific activities and isoform profiles of peroxidases, SOD, and glutathione peroxidases, along with variations in phenolic compound levels, enabled the discrimination between stressed and control seedlings of European beech. Due to higher ascorbic acid and glutathione content and slower activity loss of antioxidative enzymes involved in the decomposition of superoxide anions and hydrogen peroxide, sun-exposed leaves

showed better control over redox balance, oxidative stress, and senescence induction (Kraj and Ślepaczuk 2022). Polle et al. (1997) established that CAT activity is higher in European beech saplings with limited mineral and CO_2 supply. However, high irradiance provokes conditions of instability in the photosynthetic machinery, possibly through reactive oxygen species production at both PSII and PSI (Desotgiu et al. 2012). Lee et al. (2009) showed that activation of SOD and CAT by drought treatment continued when leaf water potential reached -1.65 MPa. After that, their activity remained stable. At the same time, guaiacol peroxidase (GPX) increased primarily under progressive drought stress and correlated with an increase in lipid peroxidation and growth restriction (Lee et al. 2009).

The role of non-enzymatic antioxidants includes reducing sugars and secondary metabolites, such as ascorbic acid, glutathione, tocopherols, carotenoids, flavonoids, phenolic compounds, proline, and anthocyanins (Ashraf et al. 2019, Rudenko et al. 2023). Specific polyphenol concentrations may serve as chemical indicators of a population's acclimation potential and could contribute to forecasting the effects of climate change (Visiné-Rajczki et al. 2023). Anthocyanins, pigments found in the vacuoles of plant cells, help absorb UV light and neutralize free radicals, particularly under light-stress conditions. Phenolic compounds, including tannins and phenolic acids, act as potent antioxidants by neutralizing reactive oxygen species (ROS) and chelating metal ions that promote oxidative stress. Flavonoids, a diverse group of polyphenolic compounds, can neutralize a wide range of ROS and protect cells from UV-induced oxidative damage (Panche et al. 2016).

Some authors (Cadahía et al. 2015)

conducted quantitative analyses that showed cinnamic acids and flavanols were the dominant components of the non-enzymatic antioxidant system in European beech leaves. The metabolomic profiling of European beech leaves identified 56 compounds, including hydroxycinnamic acid derivatives, flavan-3-ols, proanthocyanidins, and flavanols, 38 of which are cited for the first time in this species (Cadahía et al. 2015). According to Visiné-Rajczi et al. (2023), in the polyphenolic antioxidant system of beech leaves, flavonoid (quercetin, kaempferol) glycosides and flavan-3-ol compounds were shown to be potent antioxidants with a strong relation to stem growth.

One of the most vital acclimation traits of European beech to changes in illumination is its ability to develop leaves with different morphological characteristics – sun and shade ecotype leaves. This adaptation enhances light capture and maximizes photosynthetic rates in high-light environments (Wagner et al. 2010). Sun leaves in beech typically have smaller surface areas but are thicker and contain fewer chloroplasts than shade leaves (Closa et al. 2010). In addition, shaded leaves have more equal concentrations of chlorophyll *a* and chlorophyll *b*, whereas in sun leaves, this ratio favours chlorophyll *a* (Lichtenthaler et al. 2007). Fully sun-grown leaves exhibit lower and earlier maximum leaf pigment levels, faster degradation of pigments during senescence, and a higher chlorophyll *a* / chlorophyll *b* ratio compared to leaves growing deeper in the canopy (Kraj and Ślepaczuk 2022). This acclimation enables sun leaves to maximize light absorption and photosynthetic efficiency in high-light environments (Vogelmann and Gorton 2014). Eschrich et al. (1989) noted that the determination of sun and shade leaves in the winter

buds of European beech begins towards the end of July in the previous year. Sun leaves of European beech generally have higher rates of saturated photosynthesis than shade leaves. This acclimation is due to higher content and activity of Rubisco, which allows for efficient light capture and maximum carbon assimilation. On the other hand, shade leaves typically have higher light-use efficiency under low light conditions than sun leaves, thanks to thinner leaves and more chloroplasts in the mesophyll cells (Anev 2024).

This study aimed to evaluate the biochemical responses of European beech saplings to changes in light regimes following different types of forest cutting or large canopy openings caused by windthrow.

Materials and Methods

Experimental sites and plots

The biochemical responses of European beech (*Fagus sylvatica* L.) saplings to canopy openings were studied in the ‘Petrohan’ training and experimental forest range, located in the Western Stara Planina Mountains. The region has a mountainous climate with moderate continental influence, characterized by mean annual temperature of 4.8 °C at 1450 m a.s.l. and average annual precipitation of approximately 1080 mm, with higher rainfall in spring months (Kutchukova 1983, Koleva and Peneva 1990). According to Marinova and Bocheva (2023), the average temperature anomaly in Bulgaria for the reference period 1991–2020, compared to 1961–1990, is 0.8 °C. Unlike the average annual air temperature, no significant change in the annual precipitation amount was observed during this period. Marino-

va and Bocheva (2023) noted that in the Petrohan region, temperature anomalies vary between 0.5 °C in autumn and 1.5 °C in summer. At the same time, precipitation rates have decreased by an average of 10–20 mm per year, particularly in winter and spring. Soils in the area are predominantly brown acidic (district eutric cambisol), formed on granite bedrock, with average depth exceeding 80 cm (Malinova and Petrova 2019).

The experimental sites (ES) were selected along an altitude gradient in mono-dominated European beech stands with different silvicultural or disturbance histories. Each ES consisted of two plots: one with an open canopy (O) and the other with a closed canopy, which served as control (C). In 2011, the canopy of the ES1-O plot was opened through group selection harvest, where all mature 120-year-old trees (100%) were cut down, simulating a micro-disturbance in a gap of approximately 0.1 ha, which was not expanded thereafter. In 2013, the canopy of the ES2-O plot, composed of 120-year-old trees, was thinned through a progressive shelterwood

harvest over an area with a 50 m diameter, removing 40 % of the mature trees. In 2018, additional 25 % of the mature trees were removed, and the initial cutting area was expanded to 0.5 ha (Fig. 1).

In the winter of 2013/2014, an extreme wind event disturbed the 80-year-old European beech stand (ES3-O), affecting approximately 2.5 ha. All mature trees (100 %) were uprooted and subsequently removed from the windthrow area over a two-year period. In 2011, the canopy of the ES4-O plot was thinned by shelterwood harvest, with eight mature 160-year-old trees (~15 %) evenly removed across a total area of 0.1 ha. No additional trees were cut in this plot thereafter. The control C-plots were placed near the O-plots in stands that had not been subject to logging or natural disturbance for at least the last twenty years (Table 1).

Biometric indicators of the studied saplings were examined in another of our studies (Marinova and Anev 2024). Different types of forest harvesting and disturbances, combined with the altitude, slope, and aspect of the ES, result in variations

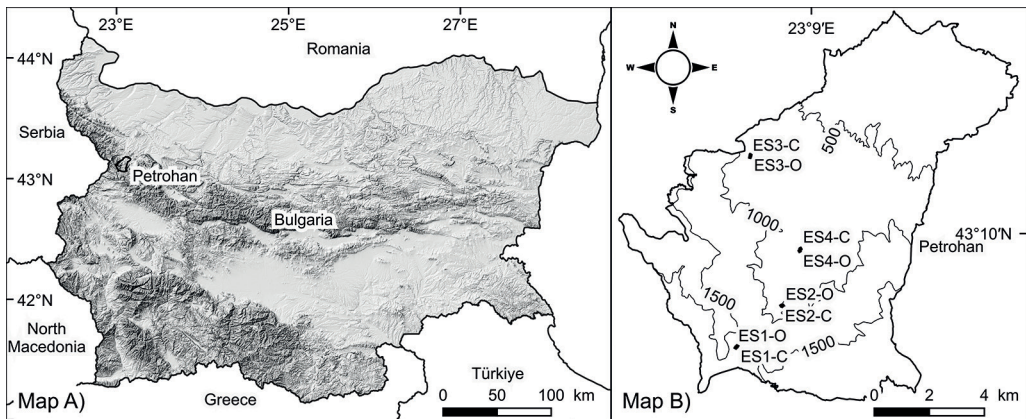


Fig. 1. Location of the experimental sites.

Note: Map A) a general view of Petrohan's position on a relief map of Bulgaria; Map B) experimental sites (ES) on a Petrohan map with elevation contour lines. CRS: WGS 84 / UTM zone 35N.

Table 1. Characteristic of the open (O) and closed (C) canopy plots in experimental sites (ES).

Plots	Latitude	Longitude	Elevation, m	Slope $\pm$ SE, °	Aspect	PPFD $\pm$ SE, $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$
ES1-C	43°7'32"	23°7'25"	1439	15.0 $\pm$ 0.8	E	47.7 $\pm$ 5.8
ES1-O	43°7'30"	23°7'23"	1450	10.9 $\pm$ 0.8	E	912.7 $\pm$ 197.3
ES2-C	43°8'22"	23°8'33"	970	24.4 $\pm$ 1.7	W	39.0 $\pm$ 12.2
ES2-O	43°8'23"	23°8'37"	1015	28.8 $\pm$ 0.4	W	773.2 $\pm$ 244.2
ES3-C	43°11'15"	23°7'32"	870	22.2 $\pm$ 2.8	E	163.1 $\pm$ 95.2
ES3-O	43°11'18"	23°7'33"	862	21.3 $\pm$ 3.0	E	1125.5 $\pm$ 186.5
ES4-C	43°9'29"	23°9'00"	713	8.4 $\pm$ 0.7	NW	171.5 $\pm$ 10.7
ES4-O	43°9'27"	23°8'59"	718	6.2 $\pm$ 1.4	NW	385.2 $\pm$ 42.2

in understory illumination. Photosynthetic photon flux density (PPFD) was measured above the crowns of saplings with a Li-190 SA portable quantum sensor (LI-COR Inc., Lincoln, NE, USA) at noon on a typical cloudless July day in the centre of the experimental sites.

Plant material and analyses

In July 2019, fifteen randomly selected leaves per plot were collected from beech saplings for biochemical analysis. Immediately after detachment, leaves were frozen in liquid nitrogen for further analysis. The activity of superoxide dismutase (SOD) was assessed following the method of Beauchamp and Fridovich (1971), which is based on the inhibition of photochemically generated superoxide anion ($\text{O}_2^{\cdot -}$) that reduces nitroblue tetrazolium to a blue-coloured formazan. Catalase (CAT) activity was determined following Aebi's method (Aebi 1984), by monitoring the decrease in absorbance of hydrogen peroxide (H_2O_2) at 240 nm, reflecting its enzymatic degradation. Guaiacol peroxidase activity was determined using the method of Hart et al. (1971), by recording the increase in absorbance at 470 nm due to the oxidation of guaiacol in the presence of H_2O_2 . The quantification of phe-

nols and flavonoids was carried out using a Shimadzu UV 1800 spectrophotometer (Kyoto, Japan), following methods described by Zhiponova et al. (2020). Total phenols were assessed using the method of Singleton et al. (1999), involving reaction with Folin-Ciocalteu reagent and sodium carbonate (Na_2CO_3), followed by absorbance measurement at 765 nm and calculation against a standard curve with gallic acid. Total flavonoids were analysed according to the method of Chang et al. (2020), involving reaction with aluminium chloride (AlCl_3) and potassium chloride (KCl), followed by absorbance measurement at 415 nm and calculation against a standard curve with quercetin. All measurements were performed in three technical replicates. The data are presented as mean $\pm$ SE. One-way analysis of variance (ANOVA) followed by Fisher's LSD test with a significance level of 0.05 was performed to estimate the differences between all variants.

Results and Discussion

Enzymatic antioxidants activity

The enzymatic antioxidant activity in the leaves of European beech saplings in-

creased in response to canopy openings and heightened light exposure. Catalase (CAT) activity was 5.18 times higher in the leaves from the windthrow ES3-O plot compared to the ES3-C plot. In ES1, this difference was 3.26 times; in ES2, it was 2.52 times; and the smallest difference, 2.39 times, was observed in ES4. This gradation closely corresponded to the size of the canopy openings and the resulting changes in the light regime. The increased CAT activity in the O-plots can be explained by higher rates of photorespiration, which produces one of the main reactive oxygen species (ROS), hydrogen peroxide (H_2O_2). Although the differences were less pronounced than those for CAT, superoxide dismutase (SOD) activity was also higher in most O-plots compared to their corresponding C-plots, except for the windthrow ES3-C plot (Fig. 2).

Although Peltzer et al. (2002) established that high temperatures can harm protective enzymes such as SOD, ascorbate peroxidase, and glutathione reductase, Polle and Rennenberg (1994) highlighted that beech leaves acclimate to high light, drought, and heat by increasing

SOD levels and activity. In our study, an increase in GPX activity in the O-plots relative to the C-plots was observed in ES1 (2.05 times higher) and especially in ES3 (3.69 times higher). In contrast, GPX levels in the C-plots of ES2 and ES4 were similarly high compared to their respective O-plots. Lee et al. (2009) noted that SOD, CAT, and GPX activity typically increases under progressive drought stress, which is likely experienced by saplings in ES1-O and ES3-O due to the complete canopy removal in these plots. However, in our experiment, such increases were not consistently observed for SOD in ES3-O. This discrepancy may be attributed to sapling exhaustion in the large windthrow area. Alternatively, it could be explained by increased SOD activity in saplings from the ES3-C plot.

Scartazza et al. (2016) observed that the total pool of ascorbate and phenols was higher in European beech leaves from the top and middle canopy layers compared to those from the bottom layer, where peroxidase activity was more prominent. Furthermore, GPX plays a critical role in phenolic synthesis (Burguieres et

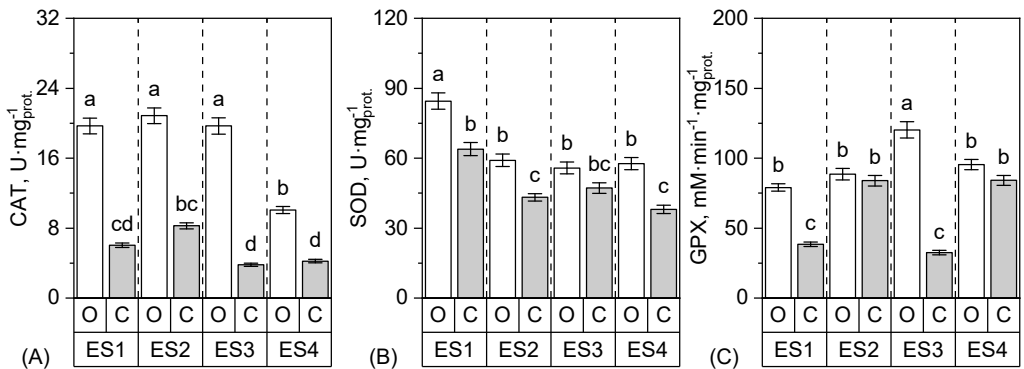


Fig. 2. Changes in the activity of enzymatic antioxidants: (A) catalase (CAT), (B) superoxide dismutase (SOD), and (C) guaiacol peroxidase (GPX) in *F. sylvatica* leaves as affected by canopy opening in the experimental sites.

Note: The data are presented as mean $\pm$ SE. Means that do not share a letter are significantly different (One-way ANOVA with Fisher's LSD test, P -value < 0.05).

al. 2007), cell wall lignification (Marjamaa et al. 2003), and the protection of auxins from oxidation and rapid degradation (Kevers et al. 1981). According to Kraj and Ślepaczuk (2022), sun leaves exhibit better control over redox balance, oxidative stress, and senescence induction. This is attributed to their higher ascorbic acid and glutathione content, as well as slower activity loss of antioxidative enzymes involved in the decomposition of superoxide anion and hydrogen peroxide (H_2O_2).

Non-enzymatic antioxidants

The content of reducing sugars was higher in all O-plots compared to their corresponding C-plots. In the O-plots, a clear trend of increasing reducing sugar accumulation with altitude was observed, except in the windthrow ES3 plot. Despite being at a lower altitude, reducing sugar levels in ES3 were equal to those in ES2. Nautiyal (1983) reported that at higher altitudes, soluble sugar content tends to be higher during the active growth phase but decreases during senescence, which may have been accelerated in ES3-O due to critically severe conditions (Anev and Marinova 2021).

Canopy opening had a lower influence on reducing sugar levels compared to altitude. The most significant differences between O- and C-plots were observed in PP2 and PP3 (1.84 times higher in O-plots), while the smallest difference was found in PP4 (1.24 times higher). In plots closer to the optimum altitude for European beech in Bulgaria (ES2 and ES3) the most substantial increases in reducing sugars after canopy opening were observed, both around 1.84 times. Conversely, at the highest (ES1) and lowest (ES4) plots, the increases were weaker: 1.35 times and 1.24 times, respectively.

Abobatta (2020) emphasized that these carbohydrates play a major role in stress mitigation, serving as carbon storage and aiding in the scavenging of reactive oxygen species under drought conditions. The similar levels of reducing sugars in ES3 and ES2, despite ES3 being 150 m lower in altitude, may indicate difficulties in assimilate transportation from the leaves or an intensified effort to enhance defence mechanisms under the drastically altered conditions of the windthrow area (Anev and Marinova 2021) (Fig. 3).

In all plots, canopy opening, whether caused by cutting or natural disturbance, increased phenol synthesis. In some plots (ES2 and ES3), flavonoid levels also increased. The differences in phenol content between O-plots and C-plots were more pronounced with larger canopy openings. For instance, phenol content in the windthrow ES3-O plot was 2.31 times higher than in ES3-C, whereas in ES4-O, where gradual short-term cutting was implemented, the increase was only 1.2 times.

A similar trend was observed for flavonoids. Significant differences between O- and C-plots were detected in ES2 and ES3, but no such differences were observed in ES1 and ES4. Visiné-Rajczi et al. (2023) noted strong correlations between proanthocyanidins, such as Procyanidin B dimer 5, and average stem diameter in European beech, suggesting that these compounds may act as growth indicators. While the low flavonoid content in ES4-O may be explained by less intense canopy thinning, another factor likely influences ES1-O. At higher altitudes, where UV exposure is greatest due to the combination of altitude and canopy opening, saplings require more UV protection.

Despite the significant synthesis of phenols at high altitudes, their transforma-

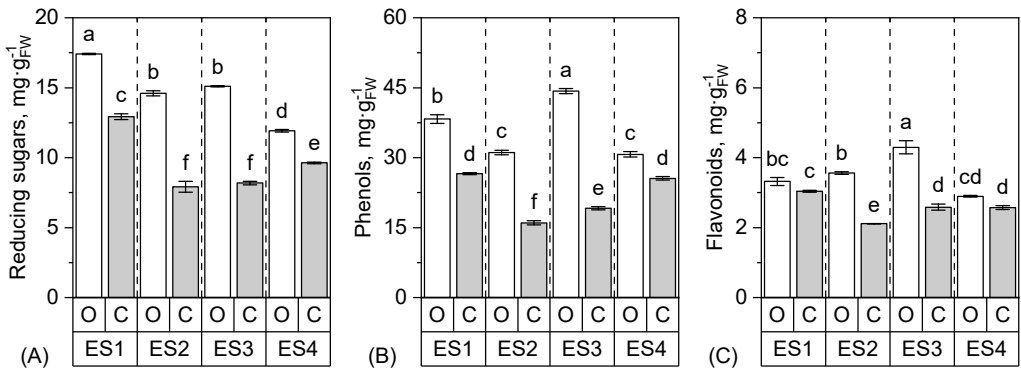


Fig. 3. Changes in the content of non-enzymatic antioxidants (A) reducing sugars; (B) phenols; and (C) flavonoids in *F. sylvatica* leaves as affected by canopy opening in experimental sites.

Note: The data are presented as mean $\pm$ SE. Means that do not share a letter are significantly different (One-way ANOVA with Fisher's LSD test, P -value < 0.05).

tion into flavonoids may be limited by an unknown factor. This suggests that different phenolic compounds might play a role in the light adaptation of European beech saplings at higher altitudes.

Photosynthetic pigments content

Site-specific variations in the amount of plastid pigments were observed. For leaves in ES1 and ES3, which were exposed to high-intensity light (Table 1), higher levels of chlorophyll *a* was measured in the C-plots than in the O-plots. The chlorophyll *a* concentration in the less illuminated ES2-O was similar to that in ES2-C. In ES4-O, which received the least change in illumination, the chlorophyll *a* concentration was even higher than in its corresponding control (ES4-C). The differences in chlorophyll *b* concentrations between the C- and O-plots were directly proportional to the degree of canopy opening. The most significant decrease in chlorophyll *b* concentration was observed in the windthrow plot ES3-O, while in the least affected plot, ES4-O, no significant

reduction in chlorophyll *b* was observed compared to the control plot. According to Vogelmann and Gorton (2014), chlorophyll *b* is the pigment that absorbs diffuse light more efficiently, and its high concentration serves as a marker of acclimation to shading. From this perspective, the lowest levels observed in the windthrow plot ES3-O could indicate a reverse acclimation process in saplings to extremely high light levels. However, these low levels may also signify exhaustion caused by the altered environment, as both chlorophyll concentrations in ES3-O were the lowest compared to all other plots. (Fig. 4).

The second O-plot, where the difference in total chlorophyll content was significant compared to the C-plot, is ES1-O, where the open gap (~ 0.1 ha) was large enough to induce similar stress. Lichtenthaler et al. (1981) indicated that high light-adapted leaves in European beech contained more chlorophyll per unit leaf area than shaded leaves, possibly due to thicker leaf laminae and more chlorophyll-bearing cells in the thicker mesophyll, especially in the palisade zone, which was

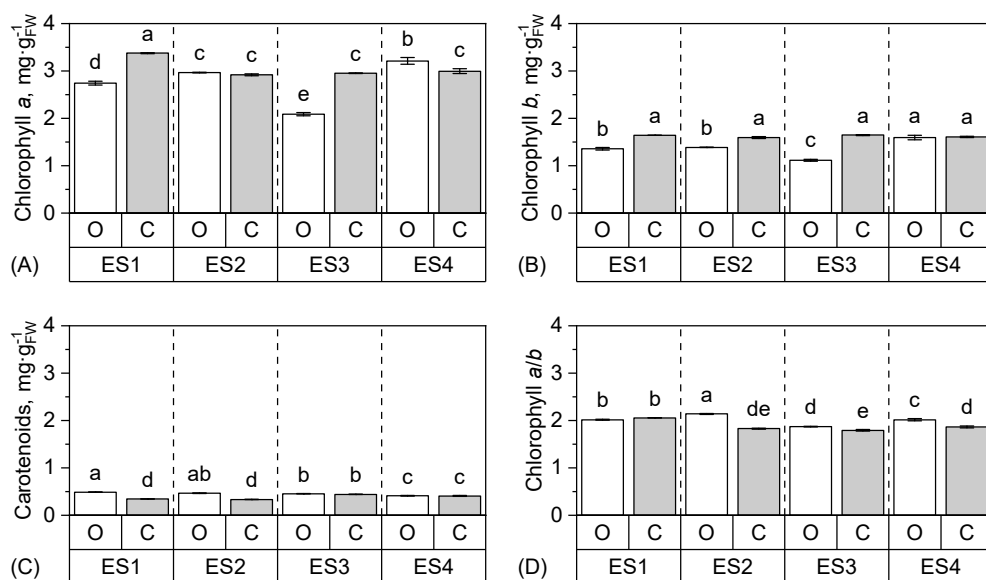


Fig. 4. Changes in the content of plastid pigments (A) chlorophyll *a*; (B) chlorophyll *b*; (C) carotenoids and (D) chlorophyll *a* / *b* ratio in *F. sylvatica* leaves as affected by canopy opening in experimental sites.

Note: The data are presented as mean $\pm$ SE. Means that do not share a letter are significantly different (One-way ANOVA with Fisher's LSD test, P -value < 0.05).

two-rowed in sunny leaves and only one-rowed in shaded leaves. However, when measured per unit leaf mass, the method used in the present experiment, shaded leaves contained more chlorophyll than sunny ones. Scartazza et al. (2016) pointed out that the chlorophyll *a* to chlorophyll *b* ratio and the pool of xanthophyll-cycle pigments in European beech leaves increased at the highest irradiance, along with lutein and β -carotene. This strategy was likely related to the need for shaded saplings to capture the scarce photons more efficiently below the canopy (Anev 2024). Our results partially confirmed this hypothesis. In ES1, ES2, and ES3, the total chlorophyll content (chlorophyll *a* + chlorophyll *b*) was significantly higher in C-plots compared to O-plots. However, in ES2, the differences were not significant (1.04 times), as in ES1 (1.22 times), with

the highest difference observed in ES3 (1.44 times). This trend was attributed to both the higher chlorophyll *a* content (1.23 and 1.41 times higher in ES1 and ES3, respectively) and the higher chlorophyll *b* content (1.21, 1.15, and 1.48 times higher in ES1, ES2, and ES3, respectively). ES4-O was the only plot where the total chlorophyll content was higher than in the corresponding C-plots.

Differences in carotenoid biosynthesis between C and O plots were observed only in ES1 and ES2, likely related to the protective function of carotenoids in neutralizing photooxidative radicals. Since carotenoids protect chlorophyll from photooxidation and photosynthesis from photoinhibition (Maoka 2020), the observed differences in ES1 and ES2 may be attributed to increased solar radiation with shorter wavelengths at higher altitudes

(Ahrens and Henson 2015). The equalized concentrations in the O and C plots in ES3 and ES4 may result from the lower UV and blue light at lower altitudes. However, in ES4, and especially in ES3, carotenoid contents might also reflect their role as signalling compounds for the synthesis of the stress hormone abscisic acid (ABA). Furthermore, Scartazza et al. (2016) concluded that non-photochemical quenching was strongly and positively related to the carotenoid / (chlorophyll $a + b$) ratio.

Conclusions

The acclimation of European beech forests to natural disturbances and human activities involved adjustments at the biochemical level, which provided a basis for physiological, anatomical, and morphological changes. Canopy openings and altered microclimates caused shifts in the saplings' metabolism and defence systems. Catalase activity increased the most after the canopy opened, helping the plant detoxify harmful hydrogen peroxide. Superoxide dismutase also increased, but to a lesser extent. Guaiacol peroxidase was significantly activated only when the saplings were fully exposed to sunlight, following the removal of shading by older trees. An increased accumulation of reducing sugars and phenolic compounds in the leaves was observed, particularly in saplings at higher altitudes or in areas with more significant canopy openings. Flavonoids also accumulated more in response to larger canopy openings, suggesting their role in plant defence against increased sunlight, especially UV radiation. The saplings in more illuminated plots had reduced chlorophyll content, particularly chlorophyll b , which captures scattered light more efficiently. At the

same time, their increased carotenoid production likely helped protect the plants from damage caused by excessive direct light. Cutting all trees during the group selection harvest practice mimics micro-disturbances and, in this regard, represents a nature-based method for regenerating beech forests. However, the signs of increased stress on saplings in ES1-O observed in the present study were similar to those seen after a large disturbance (ES3-O), suggesting the need for more careful approach to patch opening. Further studies on the influence of orography on patch location may contribute to better understanding of the mechanistic control of illumination and, thus, the stress on saplings of shade-tolerant species like European beech.

Acknowledgements

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