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Research paper

Investigating the European beech (*Fagus sylvatica* L.) leaf characteristics along the vertical canopy profile: leaf structure, photosynthetic capacity, light energy dissipation and photoprotection mechanisms

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Forest functionality and productivity are directly related to canopy light interception and can be affected by potential damage from high irradiance. However, the mechanisms by which leaves adapt to the variable light environments along the multilayer canopy profile are still poorly known. We explored the leaf morphophysiological and metabolic responses to the natural light gradient in a pure European beech (*Fagus sylvatica* L.) forest at three different canopy heights (top, middle and bottom). Structural adjustment through light-dependent modifications in leaf mass per area was the reason for most of the variations in photosynthetic capacity. The different leaf morphology along the canopy influenced nitrogen (N) partitioning, water- and photosynthetic N-use efficiency, chlorophyll (Chl) fluorescence and quali-quantitative contents of photosynthetic pigments. The Chl *a* to Chl *b* ratio and the pool of xanthophyll-cycle pigments (VAZ) increased at the highest irradiance, as well as lutein and β-carotene. The total pool of ascorbate and phenols was higher in leaves of the top and middle canopy layers when compared with the bottom layer, where the ascorbate peroxidase was relatively more activated. The non-photochemical quenching was strongly and positively related to the VAZ/(Chl *a* + *b*) ratio, while Chl *a*/Chl *b* was related to the photochemical efficiency of photosystem II. Along the multilayer canopy profile, the high energy dissipation capacity of leaves was correlated to an elevated redox potential of antioxidants. The middle layer gave the most relevant contribution to leaf area index and carboxylation capacity of the canopy. In conclusion, a complex interplay among structural, physiological and biochemical traits drives the dynamic leaf acclimation to the natural gradients of variable light environments along the tree canopy profile. The relevant differences observed in leaf traits within the canopy positions of the beech forest should be considered for improving estimation of carbon fluxes in multilayer canopy models of temperate forests.

Keywords: antioxidants, chlorophyll fluorescence, gas exchanges, leaf mass per area, light environments, photosynthetic pigments.

Introduction

Gas exchange performance and carbon (C) acquisition of temperate forest trees are the result of a long-term adaptation to nutrient supply, temperature and water availability of this zone (Niinemets and Tenhunen 1997). In such conditions, forest productivity mainly depends on light interception and tree acclimation

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to potentially damaging high irradiance (Katahata et al. 2007, Niinemets et al. 2015). Furthermore, high variation in light availability along the plant canopy and understorey is caused by modification of atmospheric clearness and of the diffusion of direct solar radiation linked to clouds, air particulates and aerosols (Farquhar and Roderick 2003, Matteucci et al. 2007, Urban et al. 2012). Light availability within the forest is extremely variable, depending on tree species, age and composition, stand density and structure, and environmental variables (García-Plazaola et al. 2004, Niinemets 2007). In this respect, forest canopies are characterized by a vertical light gradient, accompanied by relative variations in temperature and vapour pressure deficit, implying that leaves located at different heights within the canopy are variably exposed to environmental changes and stressful conditions. Light is the key element in primary production, but without an efficient transfer/transformation system, the accumulation of excess light energy can become very dangerous, provoking oxidative damages due to the over-production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (García-Plazaola et al. 2004). Moreover, in addition to light intensity, the spectral quality of light (e.g., fraction of UV radiation) to which the plants are exposed strongly influences their absorption efficiency and induction of morphophysiological changes and photoprotective mechanisms (Klem et al. 2015). Efficient light energy dissipation and photoprotection mechanisms at canopy level are essential for the optimization of light-use efficiency and the avoidance of photoinhibition and photodegradation. In particular, leaves developed within deep forest canopies are able to acclimate to different light intensity and spectral composition by morphological, physiological and biochemical changes (García-Plazaola et al. 2004, Scartazza et al. 2004, Niinemets 2007). Differences in incident irradiance between upper and lower canopy leaves are generally 20- to 50-fold for temperate forest ecosystems, but dependence of foliar physiological characteristics on light is non-linear (Niinemets et al. 1998, Niinemets 2014). Furthermore, the communities with continuous forest cover exhibit gaps varying in size distribution, resulting in a gap-understorey gradient of incident irradiance for the lower vegetation layers (Ĉater et al. 2014). As the canopy consists of high-light-exposed leaves in the upper part, shaded environments, gaps and microsites developing due to sunflecks, the total variation in light availability in such a complex structural environment is determined by spatial and temporal scales of light gradients (Niinemets and Valladares 2004, Ĉater et al. 2014). Such conditions have induced intriguing differences in forest species, which show plastic modifications in foliage photosynthetic capacity, leaf thickness (estimated as leaf mass per area (LMA), dry mass per unit area) and, consequently, the development of mesophyll, CO_2 conductance and carboxylation rate, nitrogen (N) and chlorophyll (Chl) contents (Meir et al. 2002, Montpied et al. 2009, Niinemets et al. 2015). The high acclimation capacity of canopy leaves to light variations at a timescale of days over the growing season has involved several leaf morphophysiological and biochemical changes within the canopy system; thus, many studies on different vegetation types, including temperate deciduous forests, report thicker leaves (higher LMA) at the top of the canopy and thinner leaves (lower LMA) at the bottom (Niinemets et al. 1998, Müller et al. 2001, Meir et al. 2002, García-Plazaola et al. 2004). Most of these works have investigated different plant responses to light irradiance focussing on 'sunlit' (top canopy) and 'shade' (bottom canopy) leaves, but often they did not study the vertical light gradient as a continuum under which plasticity of leaf structural and functional traits is completely expressed. On the other hand, the leaf middle layers often represent the predominant part of the canopy in terms of leaf area index (LAI) and C gain (Scartazza et al. 2004, Thornton and Zimmermann 2007). Consequently, how the morphophysiological and metabolic traits of leaves exposed to different light regimes within the canopy influence the forest functionality and productivity is still poorly known. In this context, recent studies demonstrate the need to properly integrate the photosynthetic processes within canopy gradients to improve the current state of the art on forest C gain and growth models, by scaling from the leaf to canopy level (Bonan et al. 2012, Niinemets 2014). The introduction of such vertical gradients represents an important advance from the standard 'one-big leaf' and 'two-big leaves' models, which take into account only the sunlit and shaded canopy fractions, towards the multilayer canopy models (Thornton and Zimmermann 2007, Bonan et al. 2012, Niinemets 2014). In this perspective, the study of leaf response to the variable light conditions at different layers along the vertical profile of a deep canopy, such as that of a beech (*Fagus sylvatica* L.) forest, can represent a valuable contribution to comparative studies in exploring the impact of light availability on leaf functionality in relation to canopy position. Beech is the predominant deciduous tree species in natural or close to natural forests from the lowlands to the lower mountain regions of Central and Southern Europe and Mediterranean areas (García-Plazaola and Becerril 2001, Geßler et al. 2007, Matteucci et al. 2007, Rajsnerová et al. 2015). Hence, these studies could have relevant implications for improving the existing modelling approaches for C exchange in forest ecosystems of the temperate zones.

In this work, we explored the leaf morphophysiological and metabolic traits along the vertical canopy profile of a pure beech forest of southern Europe during the growing season (mid-to-late summer), collecting leaves at the same phenological stage at three different canopy heights corresponding to top, middle and bottom layers. In addition to the upper and lower canopy leaves, few studies have evaluated the impact of variable leaf traits of the middle canopy layer on forest functionality (e.g., Scartazza et al. 2004, Iio et al. 2005). Therefore, the specific aims of our study were:

(i) to determine how leaf modifications in terms of structural traits, photosynthetic capacity, respiration and N partitioning among components of the photosynthetic apparatus vary along the vertical canopy gradient, in response to local irradiance;
(ii) to explore how structural and functional leaf traits are related to variations of energy dissipation and photoprotection mechanisms within the canopy, including Chl fluorescence, photosynthetic pigment composition and antioxidant status;
(iii) to evaluate the contribution of different canopy layers to the optimization of C uptake, water- (WUE) and photosynthetic N-use efficiency (PNUE); and
(iv) to assess how the distinction of the canopy in a top, middle and bottom fraction can be used for a better description of the forest multilayer system.

Materials and methods

Experimental site description

The experiment was carried out in a pure beech forest stand (~250 ha) near Collelongo (Abruzzo region, Central Italy) in a permanent experimental facility called 'Selva Piana' (41°50′58″N, 13°35′17″E, 1560 m elevation). The environmental and structural conditions of the stand are representative of Central Apennine beech forests. The site is equipped with a 26-m-scaffold tower hosting meteorological sensors at different heights along the canopy profile and the EUROFLUX set-up to measure the ecosystem water and CO_2 fluxes with the eddy covariance technique, as previously described (Aubinet et al. 2000, Matteucci et al. 2007). A detailed description of the site and of the stand structure has been reported in previous works (Matteucci et al. 2007, Guidolotti et al. 2013, Scartazza et al. 2013, 2015); the main characteristics of the research site are summarized in Table 1. The experiment was carried out in 2013; the amount and distribution of precipitation during the season resulted in sufficient soil water content (SWC), which never decreased below a stress level for plant functioning threshold estimated at the site (0.20 m^3 m^{-3}). The soil, a humic alisol formed on a calcareous bedrock, has a variable depth (40–100 cm). In the present study, three samplings and measurement campaigns were performed during the growing season, from the maximum leaf expansion (May–June 2013) to the beginning of leaf senescence (the end of September 2013). The dates of sampling occurred on days in which light fluctuations due to meteorological variations such as cloudy, windy and stormy events were limited; meteorological and soil conditions of the season (with indications of sampling days) are reported in Figure S1 available as Supplementary Data at *Tree Physiology* Online.

Leaf sample collection, measured light conditions and determinations of structural traits

The sample trees, located at different orientations around the scaffold tower, were selected among the co-dominant individuals within the most represented tree social classes in the studied site (34 and 60% of the total plant number and biomass, Scartazza et al. 2015). Leaves were sampled between 11:00 and 13:00 h from healthy trees (no sign of stress due to pathology or photobleaching) at different canopy height: 24 m (top, five trees), 19 m (middle, four trees) and 13 m (bottom, three trees), corresponding to three different light environments along the intra-canopy vertical profile. Measurements of photosynthetic photon flux density (PPFD) at the three canopy heights were taken between 10:00 and 15:00 h in each sampling campaign on predominantly clear days (maximum daily PPFD 1804 ± 176 μmol m^{-2} s^{-1}; for the daily means of the growing season, see Figure S1 available as Supplementary Data at *Tree Physiology* Online) and during the entire growing season (9:30–16:30 h) (Table 2). Photosynthetic photon flux density values were measured using a ceptometer (Decagon, Delta-T Devices Ltd, Cambridge, UK) oriented horizontally from the tower and positioned at each chosen height to represent as closely as possible the radiation environment of the selected plants and branches. Light measurements across the sampling campaigns for the different canopy heights were expressed as relative irradiance (RI; Table 2), i.e., the ratio of the averaged seasonal values of PPFD of the specific canopy height to incident PPFD measured above the canopy (Grassi and Bagnaresi 2001).

The LAI was measured with the LAI-2000 (LI-COR, Lincoln, NE, USA), calculated by omitting the external ring reading as in Cutini et al. (1998). The LAI of each studied canopy layer was calculated around the three measurement heights (24, 19 and

Table 1. Characteristics of the beech experimental stand (structural data from the 2012 periodic 5-year stand survey).

	Site name: Collelongo—'Selva Piana'
Lat. (N) and long. (E) coordinates	41°50′57.7″, 13°35′17.3″
Elevation (m above sea level)	1560
Annual mean air temperature (*t*, °C) (2013, 1996–2012)	7.81, 6.97
Annual total precipitation (*P*, mm) (2013, 1996–2012)	1058, 1127
Species	*Fagus sylvatica* L.
LAI (m^2 m^{-2})	5.95
Density (tree ha^{-1})	740
Age (years)[1]	122
Basal area (m^2 ha^{-1})[2]	44.2
Mean diameter (cm)[2]	25.5 ± 10.7
Mean height (m)[2]	20.7 ± 4.8
Growing wood stock (total, aboveground) (t ha^{-1})	354, 270
Annual wood increment (total, aboveground) (t ha^{-1} $year^{-1}$)	8.54, 6.21

[1]Age is calculated from data collected over 30 trees; the stand is close to even-aged.
[2]Sampling trees with trunk diameter at a breast height >2.5 cm. For diameter and height, standard deviation is reported.

Table 2. Relative irradiance, LAI and morphophysiological traits of *F. sylvatica* L. leaves collected at different canopy heights (top layer, corresponding to 24 m; middle layer, 19 m; bottom layer, 13 m). LA, average leaf area; LMA, leaf mass per area; DW, dry weight as percentage of fresh weight ((DW/FW) × 100). Values shown are means ± SE on at least three plants across the vegetative season (three sampling campaigns from June to September) for each canopy height ($n = 12$). For the determination of LA, leaf length and maximum width, 52 leaves for top layer, 40 for middle layer and 36 for the bottom layer were used. Data were analysed independently by one-way ANOVA. For each parameter, data followed by different letters in the same line are significantly different ($P \leq 0.05$, Fisher's LSD) for the significance level indicated. Before statistical analysis, an arcsin or angular transformation has been applied to data expressed as percentage.

Parameter	Canopy level			*P*-value
	Top layer	Middle layer	Bottom layer	
RI (10:00–15:00 h) sampling campaigns	0.900 ± 0.045 a	0.181 ± 0.020 b	0.017 ± 0.007 c	<0.001
RI (9:30–16:30 h) entire growing season[1]	0.885 ± 0.007 a	0.377 ± 0.018 b	0.019 ± 0.002 c	<0.001
LAI (m^2 m^{-2})	1.04 ± 0.087 c	2.80 ± 0.104 a	2.11 ± 0.121 b	<0.001
LAI fraction[2]	0.18	0.47	0.35	
LA (cm^2)	11.12 ± 1.11 b	15.29 ± 1.37 a	16.00 ± 1.21 a	0.021
Length (cm)	5.02 ± 0.29	5.68 ± 0.25	5.95 ± 0.26	0.061
Maximum width (cm)	3.19 ± 0.16 b	3.81 ± 0.20 a	3.80 ± 0.17 a	0.032
LMA (g m^{-2})	79.13 ± 3.15 a	42.68 ± 2.90 b	26.01 ± 1.14 c	<0.001
DW (%)	44.88 ± 0.97 a	41.20 ± 0.67 b	37.15 ± 0.71 c	<0.001
RWC (%)	86.64 ± 0.92	84.37 ± 1.42	82.74 ± 1.60	0.127

[1]The RI measured on several days during the entire growing season (from May to October) of the experiment year (2013).
[2]Portion of the total LAI (assumed as 1) represented by the LAI of each canopy layer analysed.

13 m) by inverting the Lambert–Beer law ($I = I_0\, e^{-kLAI}$). The depth of the single layers (top, middle and bottom) was estimated by considering their average light environment (using RI), canopy structure and features, as follows: 2.4, 6 and 12.5 m depth, respectively, either centred around the sampling height (for top and middle layers) or from 2.5 m above the ground level for the bottom layer.

In each campaign, the leaves collected from branches at different canopy heights were at the same phenological stage, avoiding changes related to leaf age. Gas exchange and morphophysiological measurements were performed on all three sampling dates, while fluorescence and biochemical determinations were concentrated in the second part of the beech vegetative cycle (samplings of August and September).

The area, length and maximum width of leaves were determined on single entire leaves (at least four leaves for each tree and canopy position) with a mean total value of 12–20 leaves for different heights for each sampling campaign, using an image analysis software (ImageJ, IJ 1.46r, http://imagej.nih.gov/ij/).

Leaf mass per area, per cent of leaf dry weight (DW, %) and leaf relative water content (RWC) were determined on discs (1.3 cm^2 of area) carefully sampled with a cork-borer from leaves (avoiding the mid-rib) used for photosynthesis measurements in the different canopy layers. Leaf discs were immediately placed in pre-weighted air-tight vials and stored at −20 °C until analysis. The vials were previously weighted to obtain the sample fresh weight (FW), after which the samples were hydrated to full turgidity for 3–4 h by floating on de-ionized water in a closed Petri dish. After hydration, the leaf samples were taken out of water, dried of any surface moisture using a tissue paper and immediately weighed to obtain the fully turgid weight (TW). Finally, leaf disks were freeze-dried and weighed to determine the DW. Relative water content was calculated as RWC (%) = [(FW − DW)/(TW − DW)] × 100, and DW (%) as (DW/FW) × 100.

Gas exchange and Chl fluorescence measurements

Gas exchange and fluorescence measurements were performed on three distinct plants located around the scaffold tower for each canopy height (24, 19 and 13 m) using the LI-6400-40 portable photosynthesis system (LI-COR) equipped with the leaf chamber fluorometer. Measurements were performed on fully expanded leaves from three individual detached branches (~80–100 cm long) for each canopy height (top, middle and bottom canopy) and sampling campaigns (June, August and September for gas exchange, $n = 9$; August and September for fluorescence parameters, $n = 6$). Each branch was cut from distinct trees; the cut end of each branch was immediately recut under water to avoid xylem embolisms and kept in the water throughout the measurements. Prior field gas exchange measurements did not show significant differences between detached and attached branches.

Instantaneous measurements of steady-state photosynthetic CO_2 assimilation rate (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), transpiration rate (E), actual photon yield of PSII photochemistry (Φ_{PSII}), Stern–Volmer non-photochemical quenching (NPQ) and the potential efficiency of PSII photochemistry (F_v/F_m) were performed between 10:00 and 15:00 h under saturating PPFD (varying between 1000 and 1800 μmol m^{-2} s^{-1} depending on the leaf position within the canopy), CO_2 concentration of 400 μmol mol^{-1} and leaf temperature ranging between 23 and 28 °C. These values were expressed as the average of the measurements made on at least three fully expanded leaves for each branch per plant for each canopy height. Measurements

of dark respiration (R_d) were also made at 400 μmol mol^{-1} of CO_2 concentration in the dark of the same leaves. Intrinsic water-use efficiency (WUE) and instantaneous WUE (iWUE) were calculated as the A to g_s and A to E ratios, respectively. Measurements of F_v/F_m were determined after at least 30 min of leaf acclimation to dark, in order to verify any presence of photoinhibitory damage.

The A/C_i curves were performed on one fully expanded leaf for each branch over a range of CO_2 concentration between 50 and 2500 μmol mol^{-1} at saturating PPFD values, using the method described by Centritto et al. (2003). Each step comprised ~5 min for adjustment and stabilization of the gas exchange parameters. Values of maximal carboxylation rate (V_{cmax}) and maximal light-driven electron transport rate (J_{max}) were estimated by fitting the mechanistic model of CO_2 assimilation proposed by Farquhar et al. (1980) to individual A/C_i response data. When necessary, measurements were corrected to 25 °C using the temperature responses of Bernacchi et al. (2001) and Bernacchi et al. (2003) for the Rubisco and RuBP-limited portions of the A/C_i curves, respectively.

Light response curves of fluorescence parameters were performed on one fully expanded leaf for each branch over a range of PPFD between 100 and 1800 μmol m^{-2} s^{-1}. Leaves were allowed to adapt to each irradiance level for ~10 min for adjustment and stabilization of the fluorescence parameters (steady-state values). Actual photon yield of PSII photochemistry in the light was determined for each PPFD value as $\Phi_{PSII} = (F'_m - F')/F'_m$ (Genty et al. 1989) at steady state, where F'_m is the maximum fluorescence yield with all PSII reaction centres in the reduced state obtained superimposing a saturating light flash during exposition to actinic light and F' is the fluorescence at the actual state of PSII reaction centres during actinic illumination. Non-photochemical quenching was determined according to the Stern–Volmer equation as $NPQ = (F_m/F'_m) - 1$, where F_m is the maximum fluorescence yield in the dark. The actual reduction state of PSII reaction centres was calculated as $1 - q_p = (F - F'_o)/(F'_m - F'_o)$, where q_p represents the photochemical quenching and F'_o is the fluorescence yield with all reaction centres open in the presence of quenching. The potential efficiency of PSII photochemistry was calculated on dark-adapted leaves as $F_v/F_m = (F_m - F_o)/F_m$, where F_o is the minimal fluorescence yield emitted by the leaves in the dark-adapted state.

Leaf N content, PNUE and partitioning within the photosynthetic apparatus

Nitrogen per leaf mass (N_m) was measured for each canopy layer on leaves of three branches from at least three plants around the tower in the August and September sampling campaigns ($n = 6$). Leaf samples were freeze dried until constant weight and subsequently ground to a fine powder. About 3–4 mg of powder was used for the determination of N_m by gas chromatography using an elemental analyser (Model NA 1500, Carlo Erba, Milan, Italy). Nitrogen per leaf area (N_a) was calculated as $N_a = N_m \times LMA$. Photosynthetic N-use efficiency was estimated as the ratio between A and N_a at saturating light intensity. The model proposed by Niinemets and Tenhunen (1997) was used to estimate the proportion of leaf N invested in Rubisco (P_R), bioenergetic pools (P_B) and light-harvesting components (P_L) from characteristic parameters of the photosynthetic response curves (V_{cmax} and J_{max}) along with information on leaf structure and pigment composition (LMA and Chl concentration).

Photosynthetic pigment analysis

Leaf pigment concentration and pattern were determined according to the method reported in Di Baccio et al. (2014). Leaf disks of known area (1.32 cm^2) were punched from leaves previously selected with the same procedure and utilized for gas exchange and Chl fluorescence measurements (August and September campaigns, $n = 6$). To assess the effect of leaf acclimation to the intra-canopy light gradient on the composition of xanthophyll-cycle pool (violaxanthin, V; anteraxanthin, A; zeaxanthin, Z) and their potential photoprotection capacity, pigment analyses were performed on dark-adapted leaves (at least 30 min) of the same detached branch used for F_v/F_m determination when all the PSII reaction centres were in the open state. Leaf disks were immediately frozen in liquid N and stored at –80 °C until analysis. Samples were ground in a mortar with liquid N and homogenized in 100% high-performance liquid chromatography (HPLC)-grade acetone in the presence of sodium ascorbate under dimmed light, filtered through 0.2-μm filters (Sartorius Stedim Biotech, Göttingen, Germany) and immediately analysed. The chromatographic separation was performed by reverse-phase HPLC using an Ultrasphere ODS column (5 μm particle size, 250 × 4.6 mm Ø; Beckman, Fullerton, CA, USA) and pigments were eluted using the following gradient of solvent A (acetonitrile/methanol, 75/25, v/v) and solvent B (methanol/ethyl acetate, 68/32, v/v): 100% A for the first 15 min, followed by a 2.5-min linear gradient to 100% B, which continued isocratically until the end of the cycle. The column was allowed to re-equilibrate in 100% solvent A for 10 min before the next injection. The separation cycle was 32 min with a flow rate of 1 cm^3 min^{-1}. Pigments were detected by their absorbance at 445 nm. To quantify the pigment content, known amounts of pure standards (Extrasynthese, Genay Cedex, France; ChromaDex, Irvine, CA, USA) were injected into the HPLC system. The VAZ pool was calculated as the sum of the xanthophyll cycle carotenoids (V + A + Z). The de-epoxidation state (DEPS) of the xanthophyll cycle in dark-adapted leaves was calculated as (Z + A)/VAZ.

Total phenol concentration

The total phenol concentration in beech leaves (August and September campaigns) was determined according to Di Baccio et al. (2008). Phenols were extracted from 400 mg of fresh leaf tissues (pooled from at least three leaves from each branch per chosen plant) by homogenization in 2.5 ml of 70% methanol for 2 h. The mixture was centrifuged twice for 5 min at 2000*g* and

the supernatant used for the Folin-Ciocalteau assay (modified from Singleton and Rossi 1965). A 30-µl aliquot of each sample was mixed with 0.150 ml of 2 N Folin-Ciocalteau reagent (Sigma-Aldrich Co., St. Louis, MO, USA), 0.60 ml of 10% Na_2CO_3 and 2.22 ml of distilled water in a 3.0 ml final volume reaction. After a 3-h incubation at room temperature in the dark, absorbance at 750 nm was measured. Gallic acid was used to prepare a standard curve, and assay results were expressed as milligram of gallic acid equivalents per gram of leaf dry mass.

Ascorbate and dehydroascorbate determination and ascorbate peroxidase assay

Ascorbate (AsA) and dehydroascorbate (DHA) determination and ascorbate peroxidase (APX) assay were performed using the method described in Di Baccio et al. (2004) with some modifications, and were carried out on beech leaves from August and September campaigns.

Aliquots of fresh leaf tissues (0.5 g) from each plant chosen (3–5) at different canopy heights were homogenized in ice-cold 5% (w/v) trichloroacetic acid, using a cold mortar and pestle. Ascorbate and total ascorbate (AsA + DHA) were determined in the supernatant after centrifugation at 15,000*g* for 10 min at 4 °C, using a method based on the reduction of ferric to ferrous ion with AsA in acid solution followed by formation of the red chelate between ferrous ion and 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline), which absorbs at 534 nm (Wang et al. 1991). Total ascorbate was determined through a reduction of DHA to AsA by 0.39 mmol l^{-1} dithiothreitol. Dehydroascorbate levels were estimated by means of the difference between total ascorbate and AsA values, and a standard curve covering the range of 0–25 nmol AsA was used.

The extraction of APX was performed in 50 mmol l^{-1} Tris–HCl, pH 7.2, containing 0.4 mmol l^{-1} Na_2EDTA; 1.0 mmol l^{-1} AsA was added to the medium to avoid inactivation during the extraction and assay. Ascorbate peroxidase activity was assayed by measuring the oxidation of AsA, operated by H_2O_2, at 290 nm and 25 °C according to Wang et al. (1991). The reaction mixture (1.0 ml final volume) was composed of 50 mmol l^{-1} potassium phosphate buffer, pH 6.6, 1.0 mmol l^{-1} AsA, 0.4 mmol l^{-1} Na_2EDTA and 50 µl of crude extract. The reaction was started by adding 0.4 mmol l^{-1} H_2O_2 to the reaction solution after a 1-min incubation at 25 °C. Corrections were made for the low, non-enzymatic oxidation of ascorbate by H_2O_2 and for the oxidation of ascorbate in the absence of H_2O_2. Activity was expressed as units (U, mmol of oxidized ascorbate per minute) per milligram of protein. Soluble protein concentration in leaf extracts was determined by the protein–dye binding method of Bradford (1976), with bovine serum albumin as standard.

Statistical analyses

The experiment was set up in a completely randomized design with at least three replicate plants for each treatment of the unique factor 'light exposure' or 'canopy height' (24, 19 and 13 m) within the vertical profile during three measurement campaigns, from June to September 2013 ($n = 6–9$). During this period, there were no significant differences in the general trend of measurements performed at the three canopy heights explored; so for each height, the replicates obtained across the season were joined. The sampling procedure and replicates for each determination were described. One-way analysis of variance (ANOVA) was applied in order to evaluate the effects of the three canopy heights on leaf traits and composition. Separation of means was performed by Fisher's least significance difference (LSD) test at a significance level of $P \leq 0.05$.

Linear regressions were used to analyse the relationships between leaf structural, physiological and/or biochemical parameters along the canopy vertical profile. Calculated significance level (P) and r^2 values of regressions are indicated on the figures whenever significant at $P \leq 0.05$.

Statistical analysis was conducted using STATISTICA 6.0, StatSoft, Inc. (Tulsa, OK, USA).

Results

Leaf structural traits, gas exchange and Chl fluorescence measurements, leaf N content and partitioning within the photosynthetic apparatus

Variations in leaf morphophysiological traits were strictly related to the decreasing RI recorded from the top to the bottom canopy layer (Table 2). Relative irradiance measured during the sampling days at the three canopy heights investigated was progressively more elevated from the bottom to the top canopy layers, varying from 0.017 (13 m) to 0.900 (24 m) through 0.181 (19 m). These data reflected the trend of RI during the entire growing season (Table 2). The highest contribution to LAI was given by the middle canopy layer, which also represented the most relevant fraction of the canopy (47%; Table 2). Both the mean surface area (LA) and the maximum width of leaves were higher in the middle (19 m) and bottom (13 m) canopy layers than in the top (24 m) one (Table 2). The LMA and the DW expressed as percentage of FW progressively increased from the bottom to the top canopy layer (1.6- to 3-fold higher for LMA and 1- to 1.2-fold higher for %DW, respectively). On the other hand, the leaf length and the RWC remained unaltered among the three canopy layers (Table 2). The LMA was highly significantly related to the light irradiance variations along the vertical canopy profile during the entire growing season ($r = 0.98$, $n = 43$, $P < 0.0001$, data not shown).

The main gas exchange and fluorescence parameters measured along the vertical canopy profile at saturating light intensity are reported in Table 3. An intra-canopy vertical gradient of A_{sat}, V_{cmax} and J_{max} was observed, with a progressive decrease of these photosynthetic parameters from the top to the bottom canopy layer. The dark respiration (R_d) was higher in the leaves from top and middle canopy layers than those from the bottom

Table 3. Gas exchange, fluorescence parameters and N content, investment in Chl and partitioning coefficients within the photosynthetic apparatus along the beech vertical canopy profile, at three different heights. Values shown are means across the vegetative season ± SE for each canopy height (n = 6–9). Statistical analysis is as in Table 2. A_{sat}, light-saturated value of CO_2 assimilation rate; g_s, stomatal conductance; C_i, intercellular CO_2 concentration, A/g_s, intrinsic water-use efficiency; Φ_{PSII}, actual efficiency of PSII photochemistry; NPQ, non-photochemical quenching; N_a, N content per unit of leaf area; N_m, N content per unit of dry mass; P_R, N partitioning in Rubisco; P_B, N partitioning in the bioenergetic pools; P_L, N partitioning in light-harvesting system components.

	Canopy level			*P*-value
	Top layer	Middle layer	Bottom layer	
Gas exchange and fluorescence				
A_{sat} (μmol m^{-2} s^{-1})[1]	8.47 ± 0.58 a	6.00 ± 0.58 b	4.36 ± 0.26 c	<0.001
g_s (mol m^{-2} s^{-1})[1]	0.110 ± 0.020	0.103 ± 0.020	0.090 ± 0.014	0.727
C_i (μmol mol^{-1})[1]	238.32 ± 14.64 b	267.87 ± 14.43 ab	289.64 ± 9.74 a	0.036
A/g_s (μmol mol^{-1})[1]	89.67 ± 10.15 a	70.22 ± 9.21 ab	54.96 ± 5.60 b	0.029
R_d (μmol m^{-2} s^{-1})[2]	1.37 ± 0.10 a	0.83 ± 0.12 a	0.59 ± 0.10 b	<0.001
V_{cmax} (μmol m^{-2} s^{-1})[3]	48.24 ± 1.84 a	35.55 ± 1.97 b	24.86 ± 1.70 c	<0.001
J_{max} (μmol m^{-2} s^{-1})[3]	143.86 ± 8.19 a	98.19 ± 5.65 b	70.02 ± 5.97 c	<0.001
V_{cmax}/J_{max}	0.342 ± 0.019	0.366 ± 0.017	0.363 ± 0.019	0.628
F_v/F_m[2]	0.808 ± 0.005	0.804 ± 0.006	0.804 ± 0.003	0.854
Φ_{PSII}[1]	0.113 ± 0.003 a	0.063 ± 0.010 b	0.049 ± 0.005 b	<0.001
NPQ[1]	3.09 ± 0.08 a	2.24 ± 0.22 b	1.87 ± 0.08 b	0.001
N determinations				
N_m (mg N g DW^{-1})	23.56 ± 1.05 b	26.69 ± 0.65 a	26.37 ± 0.97 a	0.045
N_a (g N m^{-2})	1.69 ± 0.16 a	1.08 ± 0.08 b	0.68 ± 0.04 c	<0.001
(Chl $a+b$)/N (mmol mol^{-1})	3.07 ± 0.30 b	6.49 ± 0.76 a	8.29 ± 0.63 a	<0.001
P_R	0.238 ± 0.020	0.262 ± 0.015	0.276 ± 0.040	0.304
P_B	0.069 ± 0.005	0.075 ± 0.005	0.079 ± 0.005	0.343
P_L	0.100 ± 0.009 b	0.193 ± 0.016 a	0.248 ± 0.022 a	<0.001

[1]Light-saturated values.
[2]Dark-adapted leaves.
[3]A/C_i curves at 25 °C.

layer (2.3- and 1.4-fold, respectively). Moreover, the top canopy leaves showed a higher R_d/A_{sat} ratio (0.165 ± 0.012) than the middle (0.138 ± 0.017) and bottom (0.127 ± 0.028) ones. No significant differences in A_{sat}, V_{cmax} and J_{max} were observed among the sampling campaigns, whereas R_d was significantly higher in August than in the other months (data not shown). The top canopy leaves showed lower C_i and higher A/g_s values than leaves from the bottom canopy layer, with leaves of the middle level showing intermediate values (Table 3). The stomatal conductance and V_{cmax}/J_{max} ratio did not show statistically significant differences among the analysed canopy layers.

Positive and highly significant linear relationships between LMA and both V_{cmax} and J_{max} ($r^2 = 0.88$) were observed along the canopy vertical profile (Figure 1a and b). Maximal carboxylation rate and J_{max} were also strictly related to leaf N content per unit area (N_a), whereas no significant linear relationships with leaf N content per unit of leaf dry mass (N_m) emerged from the regression analysis (Figure 1c–f).

The leaves of middle and bottom canopy layers showed higher values of N_m than the upper canopy layer, whereas a progressive decrease of leaf N_a from the top to the bottom canopy height was reported (Table 3). The Chl $a+b$ to N molar ratio was higher in the middle and bottom leaf canopy layers than in the top one, following the same trend as N_m. No significant intra-canopy differences in leaf N partitioning coefficients for Rubisco (P_R) and bioenergetic pool (P_B) components were observed. Conversely, a higher N partitioning in light-harvesting system (P_L) was observed in leaves of middle and bottom canopy layers when compared with the top leaves (Table 3).

Leaf mass per area was positively related to the iWUE (i.e., the A/E ratio) and negatively related to the PNUE along the canopy layers (Figure 2). In the first case, iWUE progressively increased from the bottom to the top leaf layer of the canopy vertical profile (Figure 2a); in the second case, PNUE in the bottom and middle layer leaves was relatively higher than in the top canopy level (Figure 2b).

The fluorescence parameters determined at different heights along the canopy indicated the highest Φ_{PSII} and NPQ values at saturating light intensity in the leaves of the top canopy layer, whereas F_v/F_m did not show any significant difference in the branches belonging to the three canopy heights (Table 3). No significant differences in fluorescence parameters emerged from the comparison between the measurement campaigns carried out in August and September (data not shown).

The light response curves of Φ_{PSII}, NPQ and $1 - q_p$ for the three different canopy heights are shown in Figure 3. The Φ_{PSII}

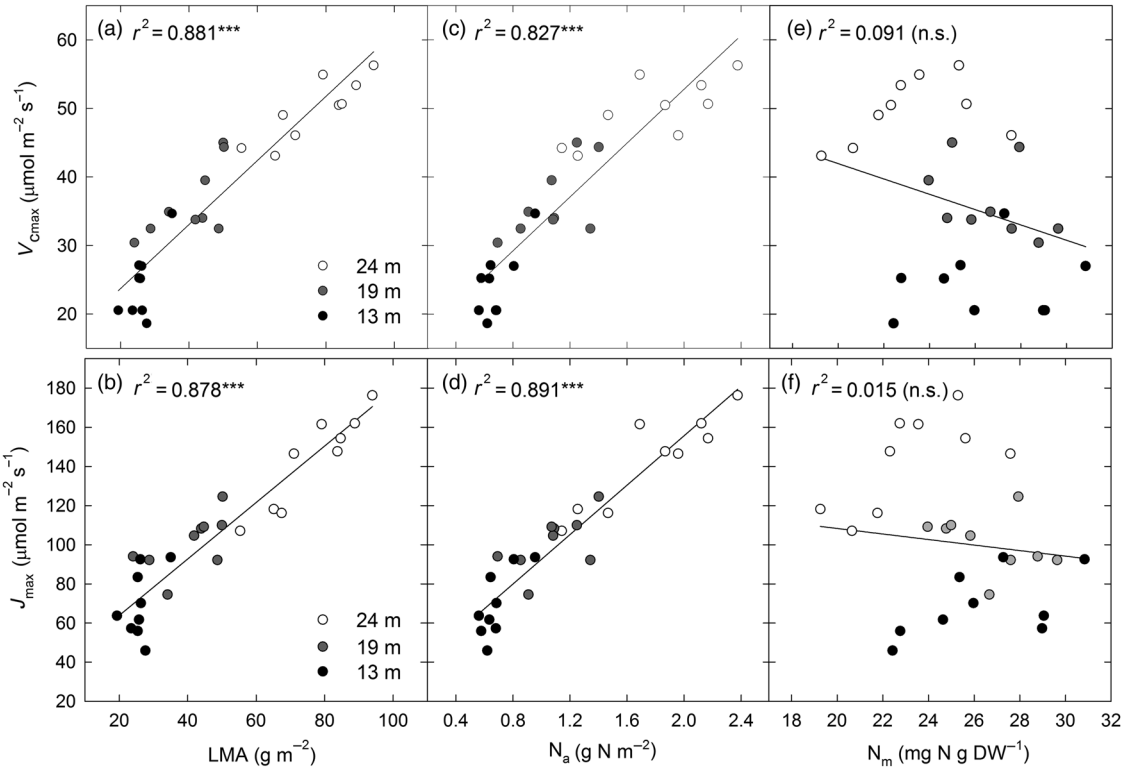

Figure 1. Relationships of V_{cmax} and J_{max} with LMA (a and b), N_a (c and d) and N_m (e and f) along the beech canopy vertical profile, at different heights (top layer, corresponding to 24 m; middle layer, 19 m; bottom layer, 13 m). Values are measurements of three campaigns across the vegetative season (June, August and September); r^2 and the significance level (***$P \leq 0.001$; n.s., not significant) of the linear regressions are shown.

decreased with the increasing PPFD in all the analysed canopy layers, although in the bottom layer (13 m), this decrease was steeper than in the upper layer especially at low PPFD values. In this condition (0–200 μmol m^{-2} s^{-1} PPFD), the reduction of Φ_{PSII} at 19 m height was similar to that observed at 24 m, while with the increasing of light irradiance, the middle layer showed an intermediate trend between those registered at 24 and 13 m (Figure 3a). Consequently, Φ_{PSII} in the top leaf canopy layer was always higher at all PPFDs with respect to the other two layers, although the differences among layers decreased with the increasing light intensity. Concurrently, NPQ showed an increase with the increasing PPFD in all the canopy layers, but with strong differences depending on height. Bottom leaves showed the highest NPQ values with respect to the middle and top ones below 200 μmol m^{-2} s^{-1} of PPFD (Figure 3b). However, over this threshold, NPQ of the top leaf layer became significantly higher in comparison with the other two canopy layers, while the bottom leaves reached a saturation value at ~400 μmol m^{-2} s^{-1} of PPFD. Differently, the middle and top canopy leaves indicated increased NPQ values until ~800 and 1200 μmol m^{-2} s^{-1} of PPFD, respectively. The reduction state of PSII increased with the increasing PPFDs in all the canopy layers. However, the top canopy leaves showed a lower $1 - q_p$ than leaves of the other two canopy layers at all PPFD values (Figure 3c). Over 800 μmol m^{-2} s^{-1} PPFD, the middle and bottom canopy layers followed similar trends for all the fluorescence parameters.

Photosynthetic pigments and leaf morphophysiological traits

When expressed on a leaf area basis, no significant differences in Chl *a*, Chl *b* and total Chl (Chl $a + b$) were observed among the studied canopy layers (Table 4). On the other hand, a progressive increase of Chl (*a*, *b* and Chl $a + b$) was revealed from the top to the bottom canopy layer, when these pigments were expressed on a leaf dry mass basis (Table 4). However, the Chl *a* to Chl *b* ratio was higher in the top leaves than in leaves from the middle and bottom canopy heights (Table 4). The lutein and VAZ contents were higher in top than in bottom leaves, whereas the middle ones showed intermediate values. The leaf content of β-carotene was 2.0- and 2.6-fold higher in the top canopy layer than in the middle and bottom ones, respectively (Table 4).

The leaf structural trait LMA from the bottom to the top layers of the canopy was negatively related to the Chl $a + b$ content per unit of dry mass and positively related to Chl *a*/Chl *b* (data not shown). The Chl *a*/Chl *b* ratio was also positively related to the light-saturated Φ_{PSII}, showing similar values in the leaves of bottom and middle canopy layers and the highest levels in the top leaves (Figure 4a). The maximum values of NPQ at saturating

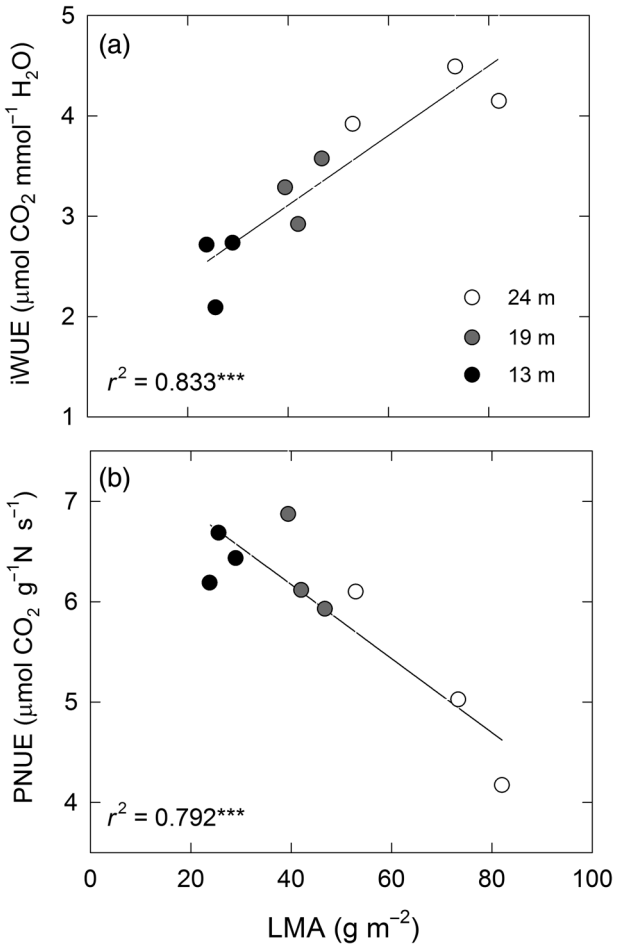

Figure 2. Relationships between LMA and iWUE (a) and PNUE (b) along the beech canopy vertical profile, at three different heights. Values represent the average of measurements made on three distinct plants for each canopy height across the vegetative season (June, August and September sampling campaigns); r^2 and the significance level (***$P \leq 0.001$) of the linear regressions are shown.

light intensity showed a positive linear relation with the VAZ/(Chl $a + b$) ratio along the canopy beech forest (Figure 4b).

Leaf antioxidant state and relations with leaf morphophysiological traits

The total (AsA + DHA) and reduced ascorbate (AsA) followed the same intra-canopy trend along the vertical profile, progressively decreasing from the top to the bottom layer (Table 5). In particular, in comparison with the top canopy leaves, AsA concentration was one-third and a half in the bottom and in the middle canopy leaves, respectively. The redox state calculated as the relative AsA to total ascorbate content in percentage was 1.2- and 1.4-fold higher in the top canopy leaves than in the middle and bottom canopy leaves, respectively. The APX activity was more elevated in the leaves of the bottom layer than in the top and middle ones (Table 5). The phenolic compounds were more concentrated in the leaves of the top than in the bottom

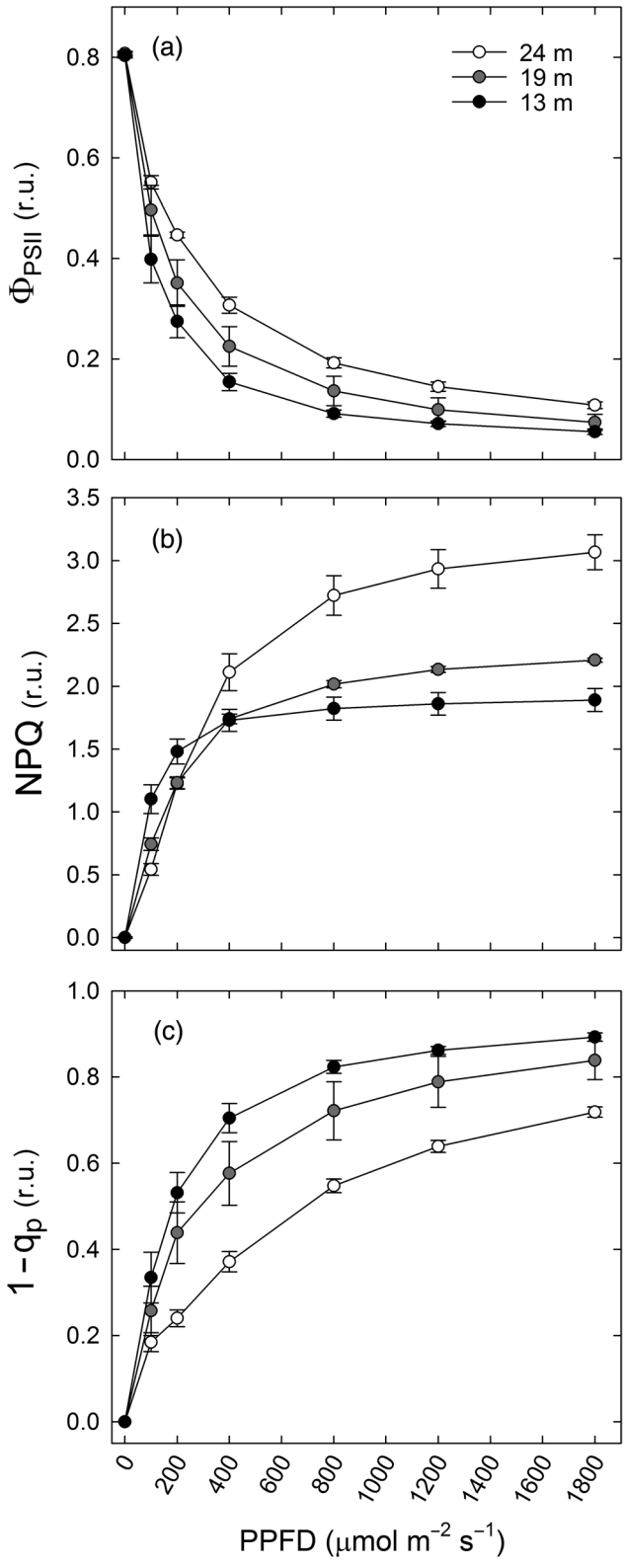

Figure 3. Light response curves of the actual photon yield of PSII photochemistry (Φ_{PSII}), Stern–Volmer NPQ and the reduction state of PSII centres ($1 - q_p$) determined along the beech canopy vertical profile, at three different heights. Values, expressed as relative units (r.u.), represent the average ± SE of two replications across the vegetative season (August and September campaigns), each calculated on three plants ($n = 6$).

Table 4. Content of leaf photosynthetic pigments of beech along the vertical canopy profile, at three different heights. Chl, chlorophyll; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; VAZ, xanthophyll-cycle pool, V + A + Z; DEPS, de-epoxidation index, (A + Z)/V + A + Z. Values represent the mean ± SE of samples collected in August and September for each canopy height ($n = 6$). Statistical analysis is as in Table 2. Before statistical analysis, an arcsin or angular transformation has been applied to the DEPS index.

Parameter	Canopy level			*P*-value
	Top layer	Middle layer	Bottom layer	
Chl *a* (μmol m^{-2})	320.02 ± 28.86	326.08 ± 20.00	296.37 ± 7.72	0.628
Chl *b* (μmol m^{-2})	105.32 ± 10.00	130.12 ± 7.93	123.83 ± 4.11	0.106
Chl *a* + *b* (μmol m^{-2})	425.34 ± 38.28	456.21 ± 27.09	420.20 ± 11.58	0.650
Chl *a* (μmol g DW^{-1})	4.19 ± 0.47 c	8.41 ± 0.85 b	11.80 ± 0.88 a	<0.001
Chl *b* (μmol g DW^{-1})	1.38 ± 0.16 c	3.38 ± 0.37 b	4.43 ± 0.36 a	<0.001
Chl *a* + *b* (μmol g DW^{-1})	5.57 ± 0.62 c	11.79 ± 1.22 b	16.73 ± 1.23 a	<0.001
Chl *a*/Chl *b*	3.06 ± 0.12 a	2.51 ± 0.08 b	2.40 ± 0.03 b	<0.001
Lutein (μmol m^{-2})	97.28 ± 9.17 a	78.51 ± 5.78 ab	68.00 ± 7.27 b	0.049
VAZ (μmol m^{-2})	46.45 ± 4.67 a	40.00 ± 4.07 ab	30.41 ± 1.53 b	0.025
VAZ/(Chl *a* + *b*)	0.102 ± 0.008 a	0.082 ± 0.007 ab	0.073 ± 0.003 b	0.019
DEPS (%) index	34.59 ± 4.20	35.41 ± 1.52	34.45 ± 4.67	0.974
β-carotene (μmol m^{-2})	45.22 ± 4.45 a	24.10 ± 4.41 b	17.07 ± 1.67 b	<0.001

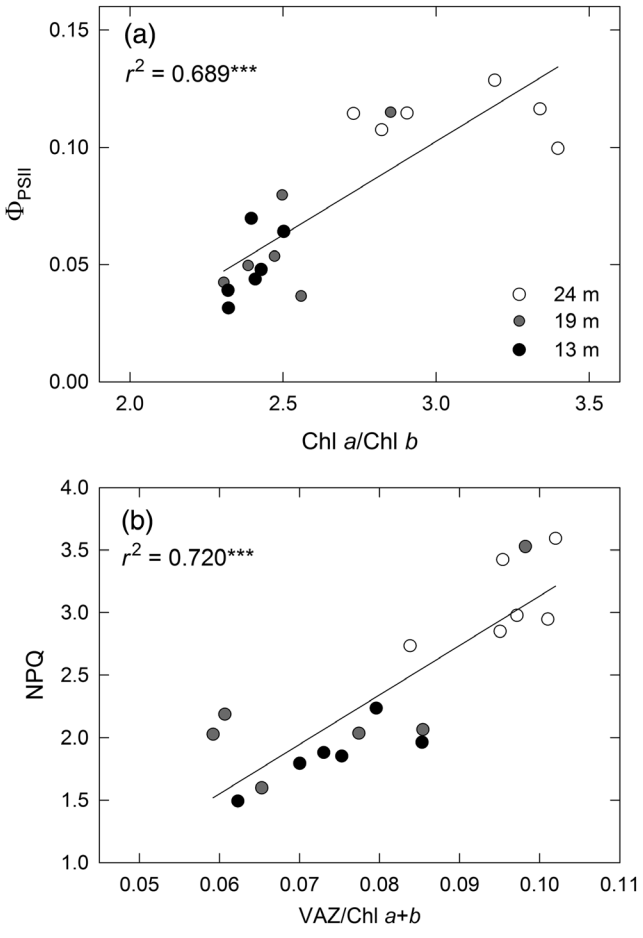

Figure 4. Relationships between Chl *a*/Chl *b* ratio and the photon yield of PSII (Φ_{PSII}, a) and between the xanthophyll pool related to total Chl molar ratio (VAZ/Chl *a* + *b*) and the NPQ (b) along the beech canopy vertical profile, at three different heights. Values are measurements of two campaigns across the vegetative season (August and September); r^2 and the significance level (***$P \leq 0.001$) of the linear regressions are shown. Actual photon yield of PSII photochemistry and NPQ were measured at saturating light intensity and photosynthetic pigments in dark-adapted leaves.

canopy layer (1.8-fold higher), whereas in the middle level, they assumed an intermediate value (Table 5).

Both leaf AsA content and its redox state were positively and highly significantly correlated to the light-saturated values of Φ_{PSII} and NPQ, with the highest values in leaves of the top canopy layer (Figure 5a–d). Ascorbate and AsA + DHA content per unit of leaf Chls were also positively and progressively (from the bottom to the top canopy layer) related to the VAZ/(Chl *a* + *b*) (Figure 5e and f).

Discussion

Leaf structural traits, photosynthetic potential and N partitioning within photosynthetic apparatus along the vertical canopy profile

The leaf structural modifications observed in the upper and lower part of the beech vertical profile were consistent with the morpho-anatomical differences in sun and shade leaves (Terashima et al. 2001, Lichtenthaler et al. 2007). The surface area of single top leaves was relatively lower than that of bottom and middle leaves, mainly due to a reduction of the lamina width (Table 2). The sun-exposed leaves of the top canopy layer showed the highest %DW and LMA, directly related to the typical larger thickness of sun leaves (by ~40–70%) when compared with the lowest light-exposed leaves, close to shade leaves (Terashima et al. 2001, Niinemets 2007). When compared with top and bottom leaves, the leaves of the middle canopy layer were characterized by a well-defined and distinct LMA value, determining a vertical gradient of this structural parameter. Moreover, LMA was strictly dependent on the intra-canopy variation of RI and hence reflected the local light environment gradient along the vertical profile (Table 2). Consequently, LMA can be used as a surrogate for the irradiance received by leaves during their growth, as proposed by other authors (e.g.,

Table 5. Concentration of reduced (AsA) and total (AsA + DHA) ascorbic acid, redox state [AsA/(AsA + DHA) × 100], APX activity and total phenolic concentration in leaves of beech along the canopy vertical profile, at three different heights. GAE, gallic acid equivalents. Values represent the mean ± SE of samples collected in August and September for each canopy height (n = 6–8). Statistical analysis is as in Table 2.

Parameter	Canopy level			P-value
	Top layer	Middle layer	Bottom layer	
AsA (µmol g DW^{-1})	23.74 ± 0.77 a	11.62 ± 1.48 b	7.73 ± 0.99 c	<0.001
AsA + DHA (µmol g DW^{-1})	32.66 ± 1.64 a	18.73 ± 2.48 b	14.58 ± 1.62 c	<0.001
Redox state	73.24 ± 2.50 a	63.00 ± 3.15 b	53.19 ± 3.45 c	<0.001
APX (U mg protein^{-1})	0.246 ± 0.017 b	0.207 ± 0.026 b	0.461 ± 0.100 a	0.049
Phenols (mg GAE g DW^{-1})	14.74 ± 2.39 a	11.56 ± 1.68 ab	8.13 ± 1.00 b	0.050

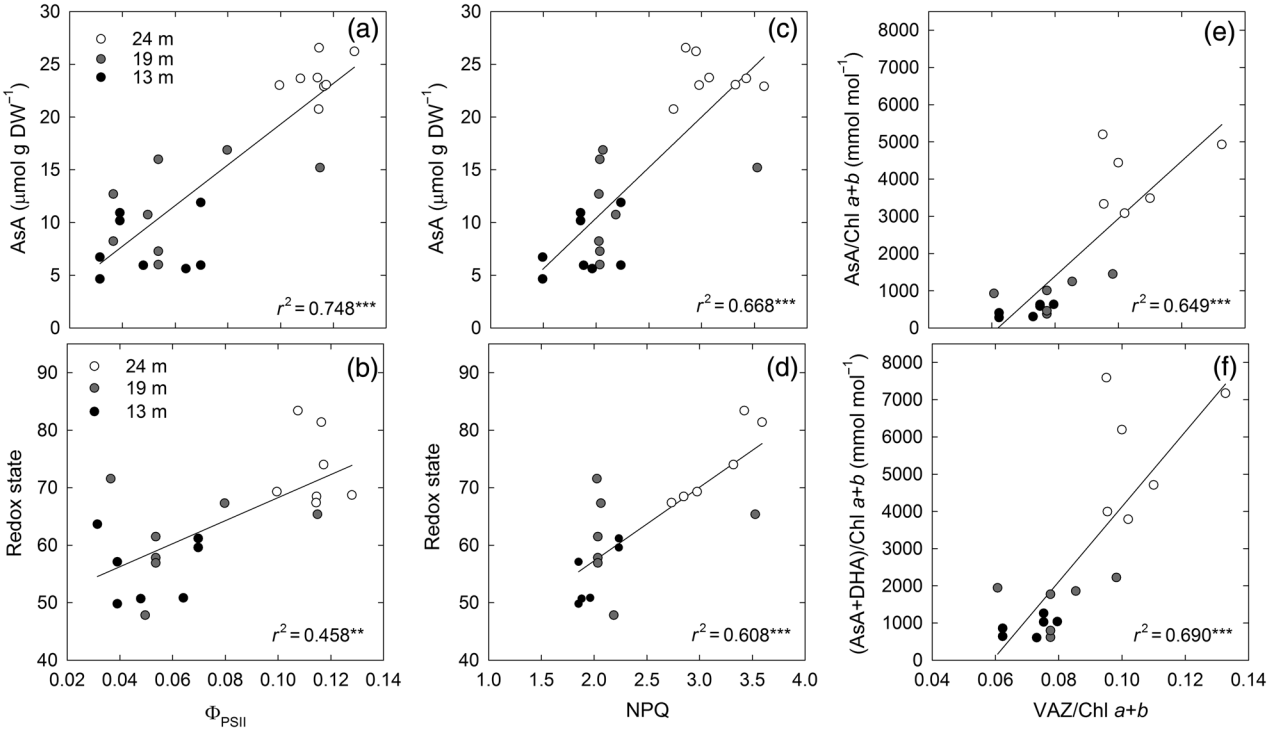

Figure 5. Relationships of leaf reduced ascorbate (AsA) and ascorbate redox state with the light-saturated values of Φ_{PSII} and NPQ (a and c; b and d, respectively), and of AsA and total (AsA + DHA) ascorbate with the xanthophyll pool (VAZ) related to Chl $a + b$ molar ratio (e and f, respectively) along the beech canopy vertical profile, at three different heights. Values are measurements of two campaigns across the vegetative season (August and September); r^2 and the significance level (**$P \leq 0.01$, ***$P \leq 0.001$) of the linear regressions are shown. Ascorbate redox state calculated as AsA/(AsA + DHA) × 100.

Niinemets and Tenhunen 1997, Montpied et al. 2009). As previously observed (Scartazza et al. 2004), the middle canopy layer gave the most relevant contribution in terms of LAI and mass (~120 g) per square metre ground area. The RWC values indicated that leaf water status was not affected by either leaf position (Table 2) or seasonal period (data not shown), confirming that during the investigated year, no water stress occurred. Indeed, SWC never fell below the threshold of drought for this beech forest (see Figure S1 available as Supplementary Data at *Tree Physiology* Online).

The light-saturated photosynthetic parameters A_{sat}, V_{cmax} and J_{max} progressively decreased from the top to the bottom canopy layer (Table 3), supporting the higher photosynthetic capacity of sun-exposed/upper canopy leaves than the shaded/lower canopy ones (Niinemets 2007, Urban et al. 2007, Montpied et al. 2009). However, for these photosynthetic parameters, the leaves of the middle canopy layer exhibited well distinguished values. The vertical variation of R_d within the canopy was consistent with physiological differences associated with sun–shade acclimation and closely coupled to changes in photosynthetic capacity along the canopy, allowing an increased C uptake of shaded leaves at low PPFDs by reducing the light compensation point (Lewis et al. 2000). Previous works showed how canopy position affects the relationships among photosynthesis, leaf respiration and associated traits (Weerasinghe et al. 2014), suggesting that the relatively higher respiratory losses and

R_d/A_{sat} ratio of the top canopy leaves might reflect their higher metabolic activity and energy demand for protein repair, maintenance of solute gradients and loading of sugars into the phloem. Our results indicated a large and continuous intra-canopy gradient of photosynthetic potential, leaf respiration and R_d/A_{sat}, strictly related to the LMA variation from the top to the bottom canopy layers (Tables 2 and 3, Figure 1). Moreover, we found a relevant role of the middle canopy layer in the forest C fluxes. The leaves of the middle layer, indeed, exhibited a higher photosynthetic potential (A_{sat}, V_{cmax} and J_{max}) compared with the bottom layer, which is associated with the highest contribution (fraction) to the LAI of the whole canopy. In this respect, the canopy-integrated mean value of V_{cmax} (34.00 μmol m^{-2} s^{-1}), calculated by weighting the V_{cmax} at the three different canopy heights with the corresponding LAI values of each specific layer, is mostly determined by the contribution of the middle canopy layer (35.55 μmol m^{-2} s^{-1}). Such intra-canopy changes of photosynthetic potential strongly affect the whole-canopy C gain (Weerasinghe et al. 2014), and could partly explain the discrepancy between observed and modelled V_{cmax} in the prediction of canopy C fluxes (Thornton and Zimmermann 2007, Bonan et al. 2012).

A central hypothesis of the Thornton and Zimmermann (2007) canopy model is that, at the species level, the vertical gradient in LMA can be represented as a linear function of LMA with LAI along the canopy vertical profile, while the mass-based leaf N concentration (N_m) shows little vertical variation or remains constant. Our results indicated that N_m was lower in the upper vs the other two canopy layers (Table 3), while the N content expressed on area basis (N_a) progressively decreased with the reduction of RI from the top to the bottom of the canopy (Meir et al. 2002, Montpied et al. 2009). Nitrogen per leaf area was not only linearly related to the light irradiance (directly linked to LMA), but also, as expected, with the V_{cmax} and J_{max} (Figure 1c and d) (Meir et al. 2002, Katahata et al. 2007). On the other hand, no significant linear relations were found between N_m and V_{cmax} or J_{max} (Figure 1e and f). These results suggest an important role of leaf N partitioning for the description of photosynthetic potential along the multilayer canopy system (Thornton and Zimmermann 2007, Bonan et al. 2012, Niinemets et al. 2015). Leaves of middle and bottom canopy layers showed a greater N partitioning to light-harvesting complexes (LHCs) (P_L) in comparison with the top canopy layer (Table 3), as observed in low-light-grown leaves of other shade-tolerant species (Niinemets and Tenhunen 1997, Grassi and Bagnaresi 2001). Moreover, the lower Chl *a*/Chl *b* ratio and greater investment of leaf N in Chl (Chl $a + b$/N) of middle and bottom leaves reflected an increase in the proportion of Chl *b* in the LHCs and a decrease in PSII complexes, suggesting acclimation to low-light environments (Niinemets et al. 1998, Katahata et al. 2007). In contrast, the N partitioning to Rubisco (P_R) and bioenergetics (P_B) did not change with the increasing light irradiance along the canopy profile (Table 3), as also reported in beech and other tree species (Niinemets and Tenhunen 1997, Niinemets et al. 1998, Valladares and Niinemets 2008). Indeed, as the photosynthetic capacity of shade and shade-acclimated leaves is dependent on rapid and frequent changes of solar irradiance, such leaves are expected to have fast photosynthetic induction kinetics, with high investment in the LHCs of the two photosystems (Valladares and Niinemets 2008). Such a hypothesis is consistent with the P_L increase in the leaves of middle and bottom canopy layers of our experiment.

The changes in V_{cmax} and J_{max} were not driven by variations in N_m but mainly by leaf structure (LMA to a large extent) and only to a lesser extent by the relative allocation of N to photosynthetic apparatus. However, since an increase in LMA resulted in a strong positive scaling of N content per area ($N_a = LMA \times N_m$), a progressive increase of N_a from the bottom to the top canopy layer was found (Table 3). Hence, LMA represented the main driving factor for changes in photosynthetic traits, as previously suggested in beech (Montpied et al. 2009) and in other shade-tolerant species (Grassi and Bagnaresi 2001).

Leaf mass per area was also positively related to iWUE and negatively related to PNUE (Figure 2), suggesting an intra-canopy gradient in water- and N-use efficiency dependent on the local light regime. The negative linear relationship between PNUE and LMA could be partly due to possible differences in internal diffusion conductance to CO_2 and fractional N distribution between photosynthetic apparatus and cell walls (Takashima et al. 2004). The higher PNUE values in the middle and bottom canopy layers were associated with the increased P_L in order to improve the light interception efficiency (Grassi and Bagnaresi 2001, Niinemets 2007). It has been observed as in the shade-tolerant species beech and small-leaved lime (*Tilia cordata* Mill.), the low-light-exposed leaves represent a high priority sink for N with an increase of light-harvesting compounds per leaf mass (Legner et al. 2014). On the contrary, the smaller investment of N in LHCs by top canopy leaves than middle and bottom ones can partly explain the lower PNUE values and the lower N_m of this higher light-exposed canopy layer (Legner et al. 2014). Hence, the leaf light-dependent structural and physiological modifications improving the photosynthetic capacity of the canopy can reduce light-harvesting efficiency and vice versa. However, the top canopy leaves, more exposed to simultaneous environmental changes of heat, water and light than bottom canopy leaves, respond more plastically to rapid variations in the light environment, demonstrating their higher acclimation capacity to multiple stresses (Niinemets 2007). For the above reasons, the top canopy layer showed higher intrinsic WUE (A/g_s) and iWUE (A/E) and a larger seasonal variability of these parameters than the bottom canopy layer, according to previous results on the same forest site (Scartazza et al. 2004, Gavrichkova et al. 2011). Furthermore, the lower PNUE and P_L and the higher R_d of the top canopy leaves in comparison with the bottom ones

suggested a minor investment of N in the photosynthetic process, possibly due to a larger investment in compounds required for longevity or defence responses related to photoprotection and antioxidant systems (Grassi and Bagnaresi 2001, García-Plazaola et al. 2004, Weerasinghe et al. 2014).

Energy dissipation mechanisms along the multilayer beech canopy profile: role of pigments and antioxidant state

Differences in leaf structural traits and photosynthetic potential along the beech canopy profile were reflected in contrasting photosynthetic efficiency and energy dissipation mechanisms. Indeed, the top canopy leaves showed the highest values of Φ_{PSII} and NPQ under saturating light intensity (Table 3, Figure 3a and b), corresponding to elevated electron transport and non-radiative energy dissipation (NRD) capacity under high radiative pressure (Müller et al. 2001, Lichtenthaler et al. 2007). On the other hand, at relatively low-light intensity (below 200 μmol m^{-2} s^{-1}), the bottom leaves showed the highest NPQ and the lowest Φ_{PSII} values (Figure 3a and b), suggesting a high capacity of this canopy layer to dissipate the excitation energy as heat in this light intensity range. Nevertheless, the high NPQ levels of bottom canopy leaves were insufficient to prevent an increase in the PSII reduction state (Figure 3c). Hence, the lower Φ_{PSII} in the bottom canopy layer can be explained not only by an increased heat dissipation, as indicated by the faster increase of NPQ between 0 and 200 μmol m^{-2} s^{-1}, but also by a rapid enhancement of PSII reduction state, as previously suggested comparing sun and shade leaves of C3 and C4 species (Brugnoli et al. 1998). However, at PPFD of ~400 μmol m^{-2} s^{-1}, the bottom leaves reached a light-saturated value for NPQ. In contrast, the NPQ of top and middle canopy leaves continued to increase with the increase of PPFD up to ~800–1200 and 1400–1800 μmol m^{-2} s^{-1}, respectively, maintaining a lower $1 - q_p$ compared with the bottom leaves. Because the C gain of shade leaves is more dependent on continuously and suddenly changing solar irradiance (Urban et al. 2007), they are expected to have faster photosynthetic induction kinetics than sun leaves. This may explain the steeper Φ_{PSII}, NPQ and $1 - q_p$ dynamic trends of bottom leaves compared with middle and top ones (Figure 3). Interestingly, the middle canopy leaves showed a similar initial trend of NPQ at relatively low PPFDs (below 200 μmol m^{-2} s^{-1}) and a lower saturation irradiance with respect to the top canopy leaves. On the other hand, the light-saturated values of NPQ, Φ_{PSII} and $1 - q_p$ of this middle canopy layer were not significantly different from those of the bottom leaves. This suggested that the middle predominant part of the forest canopy was poorly able to counteract the exposition to high irradiances through both radiative and NRD mechanisms. All these results corroborate the view that within the canopy vertical profile, we can observe a large and continuous gradient in photosynthetic and energy dissipation capacities in order to optimize the utilization of light by the multilayer canopy system.

The above-described intra-canopy variations in photosynthetic capacity and energy dissipation mechanisms were strictly related to differences in pigment composition. In particular, the Chl *a*/Chl *b* ratio in the top leaves (range: from 2.7 to 3.4) and bottom leaves (range: from 2.3 to 2.5) were typical of sun-type and shade-type chloroplasts, respectively (Brugnoli et al. 1998, Sarijeva et al. 2007); the leaves of the middle layer fell within the Chl *a*/Chl *b* range value of shade leaves (Table 4). Under low or rapid variable light irradiance within the canopy, a lower Chl *a*/Chl *b* (1.3–2.4), that is, a higher content of Chl *b*, is indicative of a large numbers of light-harvesting antenna complexes associated with the PSII (LHCII trimers), which significantly increase in size to compensate for an imbalance of light input between the two photosystems (Anderson et al. 2008, Ruban 2015). These are the light environmental conditions to which the leaves of the middle and bottom beech canopy layers were more frequently exposed (Table 2, see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Consequently, the lower Chl *a*/Chl *b* value in the middle and bottom leaves revealed an enhanced investment of Chl pigments in LHCs rather than in reactive centres in these two canopy layers, just the opposite that in the leaves of the top layer. Chlorophyll $a + b$ and the level range of Chl *a* and Chl *b* per unit of leaf area were not significantly different along the vertical canopy profile, but when expressed on a dry mass basis, all these three parameters were higher in the bottom leaves when compared with top and middle ones. In the leaves of the middle layer, the levels of Chls on dry mass were different both from top and bottom leaves; these results are partially consistent with the differences in Chl concentrations observed in shade and sun leaves (Lichtenthaler et al. 2007, Niinemets 2007, Montpied et al. 2009). This Chl pattern observed was strictly related to the progressively enhanced thickness of leaves acclimated to increasing light irradiance along the vertical canopy profile, as demonstrated by the high significant correlation between LMA and Chl $a + b$ content on a mass basis ($r = 0.904$, $P < 0.0001$, data not shown). This apparent contradiction between area- and mass-based relations of leaf photosynthetic pigments can be clarified considering that LMA is a composite variable of leaf density and thickness (Niinemets and Tenhunen 1997). Moreover, higher LMA means more layers of palisade parenchyma cells with lower and narrow thylakoidal grana stacks typical of sun-type chloroplasts with higher Chl *a*/Chl *b* ratio and less abundance of LHCII complexes (Lichtenthaler et al. 2007, 2013, Ruban 2015). This explained the positive and increasing relation observed between LMA and Chl *a*/Chl *b* from the bottom to the top of the canopy layers. The Chl *a*/Chl *b* ratio was also directly related to Φ_{PSII} at saturating light intensity, with higher values of both parameters in the top canopy leaves than in the middle and bottom ones (Figure 4a), revealing elevated electron transport capacity associated with the highest Chl *a* relative to Chl *b* content arranged in the photosystems and LHCs (Ruban 2015).

The carotenoids lutein, β-carotene and VAZ pool were more concentrated in the top canopy leaves than that in the bottom leaves, whereas the leaves of middle canopy layer contained intermediate amounts of these pigments, with the exception of β-carotene, the level of which was similar to that measured in the bottom layer (Table 4). As is known, carotenoids are bound in the pigment–protein complexes of PSI and PSII together with Chl *a* and Chl *b* (the xanthophylls lutein, neoxanthin, V, A and Z in the LHCs and β-carotene in the core complexes), increasing leaf capacity for NRD of absorbed light (Yamamoto and Bassi 1996). Furthermore, in dependence on light spectral quality and intensity, VAZ content and the conversion of V to Z via A (xanthophyll cycle) have a fundamental role in NPQ of surplus excitation energy (Demmig-Adams and Adams 2006, Klem et al. 2015). The high amount of VAZ pool in top and middle leaves revealed an enhanced photoprotection capacity against photo-oxidative damages and acclimation to high or variable light exposure of the upper and intermediate fraction of the canopy. Thus, the pool of xanthophyll cycle determines the capacity of Z formation, and several studies showed that the VAZ pool per Chl (VAZ/Chl $a+b$) or total carotenoids consistently increases with increasing absorbed light (Niinemets et al. 1998, Niinemets 2007). The enhanced VAZ size is usually associated with the increase of DEPS (Demmig-Adams and Adams 2006), while in our results, this index remained unchanged among the three canopy heights (Table 4). However, successful acclimation of beech to UV-A and UV-B can be related to increased VAZ and unaffected xanthophyll-cycle activity (Láposi et al. 2009), highlighting again the fundamental role of the spectral quality of light in its efficient absorption by plants. However, in our experiment, the pigment analyses were performed on dark-adapted leaves; thus, in this condition, the quantification of VAZ pool was more representative of the leaf potential photoprotection capacity than DEPS. Indeed, the intra-canopy trend of VAZ/(Chl $a+b$) was significantly and positively related to the NPQ at saturating light intensity (Figure 4b), supporting the central role of these xanthophylls in NRD of excess energy in leaves exposed to different light regimes and fluctuations (Niinemets et al. 1998, García-Plazaola et al. 2004, Lichtenthaler et al. 2007, 2013). Besides, recent studies show that in addition to the xanthophyll-cycle pool, lutein can contribute through the de-epoxidation reaction to the increased NPQ capacity of leaves (Förster et al. 2011). In our study, the high amount of lutein in top and middle leaves (>78.5 μmol m^{-2}) suggested a higher photoprotection potential of these leaves compared with the bottom ones due to their much larger pools of lutein and VAZ.

Plants adopt two main defence mechanisms for the dissipation of excess light energy: the thermal energy quenching mediated by pigments and the scavenging systems of ROS (Grace 2005, Demmig-Adams and Adams 2006, Noctor 2006). Reactive oxygen species detoxification is carried out by enzymatic and non-enzymatic systems based on antioxidant molecules, which can be lipophilic (carotenoids, tocopherols and terpenes) or hydrophilic (ascorbate, thiols and phenolics). Among these, we investigated the leaf phenol content and the ascorbate concentration and status, in addition to the measurement of APX activity, involved in the oxidation and re-reduction of ascorbate (Noctor and Foyer 1998). In beech leaves, the amount of phenols was 1.8-fold higher in the top canopy than in the bottom one, with intermediate values in the middle canopy leaves (Table 5). In addition to the antioxidant function, polyphenols protect leaves from excess or UV light (Mendez et al. 1999, Klem et al. 2015), partly explaining the progressive increase of these defence compounds from the bottom to the top leaves of the canopy. Leaf ascorbate (total and reduced) and its redox state showed a linear gradient, increasing from the bottom to the top canopy layer, with the middle layer exhibiting quite different values when compared with the other two (Table 5). Despite the high variability in absolute hydrophilic antioxidant amounts among different plant species, the top/bottom leaf ratio of ascorbate content found in the beech canopy was consistent with previous results in sun and shade leaves of the same species (3.3–4.8, García-Plazaola and Becerril 2001). Ascorbate has not only a direct antioxidant activity on ROS but also an indirect defence role, being a reducing equivalent of enzymes involved in the Asada–Halliwell cycle and the xanthophyll cycle (Noctor and Foyer 1998, Smirnoff 2000, Demmig-Adams and Adams 2006). Then, the higher allocation of this hydrophilic antioxidant in the leaves of the top canopy might be explained by their higher exposure to severe photo-oxidative stress in comparison with the leaves of bottom canopy layer. This observation was consistent with the strong and increasing relation of AsA and its redox state with Φ_{PSII} and NPQ at light-saturating conditions from the bottom to the top canopy layers (Figure 5a–d). Moreover, although Niinemets et al. (2003) showed that ascorbate availability is not limiting the V de-epoxidation activity, the VAZ pool in beech leaves was directly related to total and reduced ascorbate on a Chl basis, suggesting an important role of ascorbate in the turnover of the xanthophyll cycle (Figure 5e and f). The specific values of ascorbate pool and redox state assumed in the leaves of the middle canopy fraction confirmed the importance of the integration of gradients in the parameterization of models describing the canopy structure and functionality (Thornton and Zimmermann 2007, Niinemets 2014). In addition, the activity of APX was more elevated in the leaves of the bottom canopy layer than in those of the top and middle canopy layers (Table 5). This can be explained by the fact that the lower usage and dissipation of irradiance in the shade-tolerant leaves/species imply a stronger and faster protection capacity against photo-oxidative stress caused by, for example, a fleck of excess light. This prompt response of APX may be due to a higher availability of the cofactor AsA or to higher constitutive gene levels of APX (Karpinski et al. 1997, Hansen et al. 2002). In this respect, the removal of H_2O_2 by APX through the AsA oxidation was more intense in the low-light-exposed leaves compared with the leaves from top of the canopy, requiring a higher activity of this enzyme.

This might be also related to the faster depletion of AsA in the bottom canopy leaves, in which the defence against sudden oxidative burst must be particularly efficient, although the effective AsA content is the result of a complex equilibrium between its synthesis and regeneration pathways (Smirnoff 2000, Noctor 2006). All these results support the importance of the interplay between leaf content and organization of photosynthetic pigments and antioxidant compounds in the plant responsiveness to light gradients along the canopy profile.

Conclusions

Our investigations in a monospecific beech site represent a valuable example of the Mediterranean forested area, and hence could be useful in comparative studies for exploring how the natural light gradients influence the canopy structure and functionality along the vertical profile in such temperate zones. We found that the main driving factors for changes in leaf photosynthetic performance are structural traits such as LMA, N and pigment contents, on which the plastic metabolic response of the canopy layers to different light irradiances depends. The impact of light irradiance on photosynthetic capacity was also determined by the development of antioxidative defence systems, which combine the dissipation of excess light energy and the scavenging of increased ROS through the interaction between lipophilic and hydrophilic antioxidant molecules and enzymes such as carotenoids, phenols, ascorbate and APX. The light-saturated values of PSII photochemical efficiency and NRD mechanisms were mediated by pigment composition (Chl *a*/Chl *b*, lutein, VAZ/(Chl *a* + *b*) and β-carotene) and antioxidant capacity (phenolic compounds, reduced ascorbate and its redox state). All these parameters changed in a continuum along the vertical canopy profile depending on the leaf canopy position, with a relevant contribution of the middle canopy layer to the LAI, V_{cmax} and specific photoprotection responses. Results revealed how the plastic response of the canopy to extremely variable conditions of light environments is influenced by the leaf position along the multilayer canopy profile through the synergistic role of photoprotection and antioxidant systems. The intra-canopy profiles of leaf traits, photosynthetic potential and excess light dissipation mechanisms observed in this study provide complementary information for improving the parameterization of leaf traits in canopy C flux models when simulating vegetation C gain and canopy scaling leaf processes. Moreover, our results obtained at the leaf scale can contribute to the understanding of C gain variations and light dissipation and photoprotection mechanisms at canopy level in broadleaf forests of Mediterranean areas.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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References

Anderson J, Chow WS, De Las Rivas J (2008) Dynamic flexibility in the structure and function of photosystem II in higher plant thylakoid membranes: the grana enigma. Photosynth Res 98:575–587.

Aubinet M, Grelle A, Ibrom A et al. (2000) Estimates of the annual net carbon and water exchange of forests: the EUROFLUX methodology. Adv Ecol Res 30:113–175.

Bernacchi CJ, Singsaas EL, Pimentel C, Portis AR Jr, Long SP (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. Plant Cell Environ 24:253–259.

Bernacchi CJ, Pimentel C, Long SP (2003) *In vivo* temperature response functions of parameters required to model RuBP-limited photosynthesis. Plant Cell Environ 26:1419–1430.

Bonan GB, Oleson KW, Fisher RA, Lasslop G, Reicstein M (2012) Reconciling leaf physiological traits and canopy flux data: use of the TRY and FLUXNET databases in the Community Land Model version 4. J Geophys Res 117:G02026. doi:10.1029/2011JG001913

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Brugnoli E, Scartazza A, De Tullio MC, Monteverdi MC, Lauteri M, Augusti A (1998) Zeaxanthin and non-photochemical quenching in sun and shade leaves of C_3 and C_4 plants. Physiol Plant 104:727–734.

Ĉater M, Diaci J, Roženbergar D (2014) Gap size and position influence variable response of *Fagus sylvatica* L. and *Abies alba* Mill. Forest Ecol Manag 325:128–135.

Centritto M, Loreto F, Chartzoulakis K (2003) The use of low [CO_2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. Plant Cell Environ 26:585–594.

Cutini A, Matteucci G, Scarascia Mugnozza G (1998) Estimation of leaf area index with the Li-Cor LAI 2000 in deciduous forests. Forest Ecol Manag 105:55–65.

Demmig-Adams B, Adams WW III (2006) Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. New Phytol 172:11–21.

Di Baccio D, Navari-Izzo F, Izzo R (2004) Seawater irrigation: antioxidant defence responses in leaves and roots of a sunflower (*Helianthus annuus* L.) ecotype. J Plant Physiol 161:1359–1366.

Di Baccio D, Castagna A, Paoletti E, Sebastiani L, Ranieri A (2008) Could the differences in O_3 sensitivity between two poplar clones be related to a difference in antioxidant defense and secondary metabolic response to O_3 influx? Tree Physiol 28:1761–1772.

Di Baccio D, Castagna A, Tognetti R, Ranieri A, Sebastiani L (2014) Early responses to cadmium of two poplar clones that differ in stress tolerance. J Plant Physiol 171:1693–1705.

Farquhar GD, Roderick ML (2003) Pinatubo, diffuse light, and the carbon cycle. Science 299:1997–1998.

Farquhar GD, von Caemmerer S, Berry JA (1980) A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149:78–90.

Förster B, Pogson BJ, Osmond CB (2011) Lutein from deepoxidation of lutein epoxide replaces zeaxanthin to sustain an enhanced capacity for nonphotochemical chlorophyll fluorescence quenching in avocado shade leaves in the dark. Plant Physiol 156:393–403.

García-Plazaola JI, Becerril JM (2001) Seasonal changes in photosynthetic pigments and antioxidants in beech (*Fagus sylvatica*) in a Mediterranean climate: implications for tree decline diagnosis. Aust J Plant Physiol 28:225–232.

García-Plazaola JI, Becerril JM, Hernández A, Niinemts Ü, Kollist H (2004) Acclimation of antioxidant pools to the light environment in a natural forest canopy. New Phytol 163:87–97.

Gavrichkova O, Proietti S, Moscatello S, Portarena S, Battistelli A, Matteucci G, Brugnoli E (2011) Short-term natural $\delta^{13}C$ and $\delta^{18}O$ variations in pools and fluxes in a beech forest: the transfer of isotopic signal from recent photosynthates to soil respired CO_2. Biogeosciences 8:2833–2846.

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta Gen Subj 990:87–92.

Geßler A, Keitel C, Kreuzwieser J, Matyssek R, Seiler W, Rennenberg H (2007) Potential risks for European beech (*Fagus sylvatica* L.) in a changing climate. Trees 21:1–11.

Grace SC (2005) Phenolics as antioxidants. In: Smirnoff N (ed.) Antioxidants and reactive oxygen species in plants. Blackwell Scientific Publishers, Oxford, UK, pp 141–168.

Grassi C, Bagnaresi U (2001) Foliar morphological and physiological plasticity in *Picea abies* and *Abies alba* saplings along a natural light gradient. Tree Physiol 21:959–967.

Guidolotti G, Rey A, D'Andrea E, Matteucci G, De Angelis P (2013) Effect of environmental variables and stand structure on ecosystem respiration components in a Mediterranean beech forest. Tree Physiol 33:960–972.

Hansen U, Fiedler B, Rank B (2002) Variation of pigment composition and antioxidative systems along the canopy light gradient in a mixed beech/oak forest: a comparative study on deciduous tree species differing in shade tolerance. Trees 16:354–364.

Iio A, Fukasawa H, Nose Y, Kato S, Kakubari Y (2005) Vertical, horizontal and azimuthal variations in leaf photosynthetic characteristics within a *Fagus crenata* crown in relation to light acclimation. Tree Physiol 25:533–544.

Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. Plant Cell 9:627–640.

Katahata S-I, Naramoto M, Kakubari Y, Mukai Y (2007) Photosynthetic capacity and nitrogen partitioning in foliage of the evergreen shrub *Daphniphyllum humile* along a natural light gradient. Tree Physiol 27:199–208.

Klem K, Holub P, Štroch M, Nezval J, Špunda V, Tříska J, Jansen MAK, Robson TM, Urban O (2015) Ultraviolet and photosynthetically active radiation can both induce photoprotective capacity allowing barley to overcome high radiation stress. Plant Physiol Biochem 93:74–83.

Láposi R, Veres S, Lakatos G, Oláh V, Fieldsend A, Mészáros I (2009) Responses of leaf traits of European beech (*Fagus sylvatica* L.) saplings to supplemental UV-B radiation and UV-B exclusion. Agric For Meteorol 149:745–755.

Legner N, Fleck S, Leuschner C (2014) Within-canopy variation in photosynthetic capacity, SLA and foliar N in temperate broad-leaved trees with contrasting shade tolerance. Trees 28:263–280.

Lewis JD, McKane RB, Tingey DT, Beedlow PA (2000) Vertical gradients in photosynthetic light response within an old-growth Douglas-fir and western hemlock canopy. Tree Physiol 20:447–456.

Lichtenthaler HK, Alexander Ač, Marek MV, Kalina J, Urban O (2007) Differences in pigment composition, photosynthetic rates and chlorophyll fluorescence images of sun and shade leaves of four tree species. Plant Physiol Biochem 45:577–588.

Lichtenthaler HK, Babani F, Navrátil M, Buschmann C (2013) Chlorophyll fluorescence kinetics, photosynthetic activity, and pigment composition of blue-shade and half-shade leaves as compared to sun and shade leaves of different trees. Photosynth Res 117:355–366.

Matteucci G, Masci A, Valentini R, Scarascia G (2007) The response of forests to global change: measurements and modelling simulations in a mountain forest of the Mediterranean region. In: Palahi M, Byrot Y, Rois M (eds) Scientific tools and research needs for multifunctional Mediterranean forest ecosystem management. Vol. 56. EFI Proceedings, Joensuu, Finland, pp 11–23. http://www.efi.int/portal/virtual_library/publications/proceedings/56/ (11 May 2016, date last accessed).

Meir P, Kruijt B, Broadmeadow M, Barbosa E, Kull O, Carswell F, Nobre A, Jarvis PG (2002) Acclimation of photosynthetic capacity to irradiance in tree canopies in relation to leaf nitrogen concentration and leaf mass per unit area. Plant Cell Environ 25:343–357.

Mendez M, Jones DG, Manetas Y (1999) Enhanced UV-B radiation under field conditions increases anthocyanin and reduces the risk of photoinhibition but does not affect growth in the carnivorous plant *Pinguicula vulgaris*. New Phytol 144:275–282.

Montpied P, Granier A, Dreyer E (2009) Seasonal time-course of gradients of photosynthetic capacity and mesophyll conductance to CO_2 across a beech (*Fagus sylvatica* L.) canopy. J Exp Bot 60:2407–2418.

Müller P, Li X-P, Niyogi KK (2001) Non-photochemical quenching. A response to excess light energy. Plant Physiol 125:1558–1566.

Niinemets Ü (2007) Photosynthesis and resource distribution through plant canopies. Plant Cell Environ 30:1052–1071.

Niinemets Ü (2014) Improving modeling of the 'dark part' of canopy carbon gain. Tree Physiol 34:557–563.

Niinemets Ü, Tenhunen JD (1997) A model separating leaf structural and physiological effects on carbon gain along light gradients for the shade-tolerant species *Acer saccharum*. Plant Cell Environ 20: 845–866.

Niinemets Ü, Valladares F (2004) Photosynthetic acclimation to simultaneous and interacting environmental stresses along natural light gradients: optimality and constraints. Plant Biol 6:254–268.

Niinemets Ü, Bilger W, Kull O, Tenhunen JD (1998) Acclimation to high irradiance in temperate deciduous trees in the field: changes in xanthophyll cycle pool size and in photosynthetic capacity along a canopy light gradient. Plant Cell Environ 21:1205–1218.

Niinemets Ü, Kollist H, García-Plazaola JI, Hernández A, Becerril JM (2003) Do the capacity and kinetics for modification of xanthophyll cycle pool size depend on growth irradiance in temperate trees? Plant Cell Environ 26:1787–1801.

Niinemets Ü, Keenan TF, Hallik L (2015) A worldwide analysis of within-canopy variations in leaf structural, chemical and physiological traits across plant functional types. New Phytol 205:973–993.

Noctor G (2006) Metabolic signalling in defence and stress: the central roles of soluble redox couples. Plant Cell Environ 29:409–425.

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249–279.

Rajsnerová P, Klem K, Holub P et al. (2015) Morphological, biochemical and physiological traits of upper and lower canopy leaves of European beech tend to converge with increasing altitude. Tree Physiol 35:47–60.

Ruban AV (2015) Evolution under the sun: optimizing light harvesting in photosynthesis. J Exp Bot 66:7–23.

Sarijeva G, Knapp M, Lichtenthaler HK (2007) Differences in photosynthetic activity, chlorophyll and carotenoid levels, and in chlorophyll fluorescence parameters in green sun and shade leaves of *Ginkgo* and *Fagus*. J Plant Physiol 164:950–955.

Scartazza A, Mata C, Matteucci G, Yakir D, Moscatello S, Brugnoli E (2004) Comparisons of $\delta^{13}C$ of photosynthetic products and ecosystem respiratory CO_2 and their responses to seasonal climate variability. Oecologia 140:340–351.

Scartazza A, Moscatello S, Matteucci G, Battistelli A, Brugnoli E (2013) Seasonal and inter-annual dynamics of growth, non-structural carbohydrates and C stable isotopes in a Mediterranean beech forest. Tree Physiol 33:730–742.

Scartazza A, Moscatello S, Matteucci G, Battistelli A, Brugnoli E (2015) Combining stable isotope and carbohydrate analyses in phloem sap and fine roots to study seasonal changes of source–sink relationships in a Mediterranean beech forest. Tree Physiol 35:829–839.

Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am J Enol Vitic 16:144–158.

Smirnoff N (2000) Ascorbate biosynthesis and function in photoprotection. Philos Trans R Soc B 355:1455–1464.

Takashima T, Hikosaka K, Hirose T (2004) Photosynthesis or persistence: nitrogen allocation in leaves of evergreen and deciduous *Quercus* species. Plant Cell Environ 27:1047–1054.

Terashima I, Miyazawa S-I, Hanba YT (2001) Why are sun leaves thicker than shade leaves? — Consideration based on analyses of CO_2 diffusion in the leaf. J Plant Res 114:93–105.

Thornton PE, Zimmermann NE (2007) An improved canopy integration scheme for a land surface model with prognostic canopy structure. J Climate 20:3902–3923.

Urban O, Košvancová M, Marek MV, Lichtenthaler HK (2007) Induction of photosynthesis and importance of limitations during the induction phase in sun and shade leaves of five ecologically contrasting tree species from the temperate zone. Tree Physiol 27:1207–1215.

Urban O, Klem K, Ač A et al. (2012) Impact of clear and cloudy sky conditions on the vertical distribution of photosynthetic CO_2 uptake within a spruce canopy. Funct Ecol 26:46–55.

Valladares F, Niinemets Ü (2008) Shade tolerance, a key plant feature of complex nature and consequences. Annu Rev Ecol Syst 39:237–257.

Wang SY, Jiao HJ, Faust M (1991) Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple. Physiol Plant 82:231–236.

Weerasinghe LK, Creek D, Crous KY, Xiang S, Liddell MJ, Turnbull MH, Atkin OK (2014) Canopy position affects the relationships between leaf respiration and associated traits in a tropical rainforest in Far North Queensland. Tree Physiol 34:564–584.

Yamamoto HY, Bassi R (1996) Carotenoids: localization and function. In: Ort DR, Yocum CF (eds) Advances in photosynthesis—oxygenic photosynthesis: the light reactions. Vol. 4. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 539–563.