Acclimation of antioxidant pools to the light environment in a natural forest canopy

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Summary

• Leaf growth irradiance determines the pools of photoprotective molecules. We asked whether the potential for acclimation of antioxidant pool size to changes in the leaf light environment is affected by the position of the leaf within the canopy profile.

• The study was conducted in a mixed canopy formed by Tilia cordata at the lower level and Populus tremula at the upper level. Leaves were either exposed to extra light or enclosed in shade bags.

• Ascorbate, glutathione and α-tocopherol pools increased with growth irradiance. Only α-tocopherol increased in leaves of both species in response to extra light. The slope of tocopherol changes was positively correlated with growth irradiance in both species. It also correlated with the slope of xanthophyll cycle (VAZ, sum of violaxanthin, antheraxanthin and zeaxanthin) pool changes with cumulative extra light in T. cordata.

• We conclude that α-tocopherol is the key antioxidant altering tolerance to high light, and that it may cooperate with zeaxanthin. The pools of hydrophilic antioxidants either acclimate more slowly, or their pools are large enough not to limit the overall acclimation to altered light environment.

Key words: antioxidants, ascorbate, canopy light gradient, glutathione, light acclimation, Populus tremula, Tilia cordata, tocopherol.

Introduction

Forest canopies are characterized by a vertical light gradient. Leaves within these canopies are able to acclimate to different light environments through a set of morphological, physiological and biochemical changes. These changes tend to optimize the photosynthetic process under light-limited conditions and enhance the protective capacity to excess light in sun-exposed sites (Valladares & Pugnaire, 1999). Both situations represent the extremes of the continuous canopy light gradient which results in development of leaves with a different potential for dissipation and light harvesting within the forest canopy profile. Thus an adjustment to the light gradient of photosynthetic capacity is observed between leaves (Hirose & Werger, 1987), as well as an increased photoprotection in the outermost layers. Superior photoprotection may be achieved through the structural avoidance of light interception (Valladares & Pearcy, 1998) or through the physiological mechanisms that increase the rate of energy dissipation or photon usage in photosynthesis.

Dissipation of excess light energy proceeds by two main biochemical mechanisms: xanthophyll cycle (VAZ, sum of violaxanthin, antheraxanthin and zeaxanthin) dependent thermal energy dissipation (recently reviewed by Demmig-Adams et al., 1999; Niyogi, 2000; Müller et al., 2001) and the scavenging of reactive oxygen species (ROS) that result from over-excitation of the photosynthetic apparatus (Mittler, 2002). Detoxification of ROS is carried out by several enzymatic mechanisms and through a system of physiological antioxidant molecules, which can be lipophilic (tocopherols, carotenoids, terpenes) or hydrophilic (ascorbate, thiols, phenolics). The latter can act as direct scavengers of ROS, but are also reductants in some of the enzymatic reactions involved in photoprotection, as is the case for glutathione peroxidase,
ascorbate peroxidase and violaxanthin de-epoxidase (Foyer & Harbinson, 1999; Mittler, 2002). On the other hand, lipophilic antioxidants are able to deactivate ROS, but also to terminate lipid peroxidation chains and to prevent the formation of a chlorophyll triplet excited state (Niyogi, 2000; Mittler, 2002; Munné-Bosch & Alegre, 2002b). Lipophilic and hydrophilic antioxidants do not operate independently, and, for example, ascorbate is also involved in the regeneration of α-tocopheroxyl radicals that result from α-tocopherol oxidation (Munné-Bosch & Alegre, 2002a).

Despite the important interplay of the antioxidant molecules, simultaneous responses of lipophilic and hydrophilic antioxidants to the light environment of natural forest canopies have been studied in only a few tree species (Schwanz et al., 1996; García-Plazaola et al., 1999; García-Plazaola & Becerril, 2001; Hansen et al., 2002; García-Plazaola et al., 2003a; Hansen et al., 2003; Wieser et al., 2003). Moreover, only two of these works have described the patterns of photoprotective compounds through a vertical forest light gradient (Hansen et al., 2002; Hansen et al., 2003), while most have focused on ‘sun’ and ‘shade’ leaves that represent arbitrarily chosen points along the continuous light gradient. Consistent with a higher demand for photoprotection at higher irradiances, all these works show that antioxidant pools are generally several-fold higher in sun leaves as compared with shade leaves. Other reports have conclusively demonstrated that the pool of xanthophyll cycle pigments (VAZ) also increased with growth irradiance (Logan et al., 1996; Logan et al., 1998a; Niinemets et al., 1999).

In natural conditions, the light environment is far from being stable. Photon flux densities fluctuate during the day because of changes in solar incidence angle, cloud cover and heterogeneous distribution of canopy gaps that leads to sunflecks of varying duration and intensity (Pearcy, 1990). Creation of canopy gaps may further generate sudden increases in irradiance (Denslow, 1987). As absorbed energy that is not used in photosynthesis or dissipated as heat may lead to oxidative damage, dissipation and absorption mechanisms must be plastic enough to compensate quickly for changed irradiance conditions. In strongly fluctuating environments the species with higher acclimation capacity would be favoured (Gamper et al., 2000; Yamashita et al., 2000). We have previously observed a significant effect of earlier long-term leaf irradiance on adjustments of the VAZ pool size after changes in light conditions (Niinemets et al., 2003). In this work we showed that after 11 d extra illumination, VAZ per area increased in upper canopy leaves and remained constant or decreased in lower-canopy leaves, while foliar chlorophyll contents of exposed leaves declined significantly more in the upper canopy. These results are summarized in Fig. 1.

Although there is a consensus that light-dependent modifications of water- and lipid-soluble antioxidants are qualitatively the same as those in the xanthophyll cycle carotenoids, few investigations have been directly conducted in the field.

To our knowledge, only Logan et al. (1998b) and Niinemets et al. (2003) have studied the capacity for modification of the VAZ pool size in response to a sudden change in irradiance in the field, and only Logan et al. (1998b) have investigated the potential for reacclimation of antioxidant pools of leaves to a modified light environment. Such studies are required as plant acclimation responses to fluctuating natural irradiances may differ significantly from the responses observed in constant environmental conditions in growth chambers, especially because multiple environmental factors interact with light in the field (Niinemets et al., 2003; Niinemets & Valladares, 2004). Furthermore, even in controlled growth chamber experiments, the whole array of antioxidant compounds has been studied in few cases.

In the current study we analyse the dynamic acclimation of the hydrophilic antioxidants ascorbate and glutathione, and the lipid-soluble antioxidants (β + γ), δ- and α-tocopherols, to a change in irradiance through the light gradient of a mixed
canopy formed by a shade-tolerant species (*Tilia cordata*) and a shade-intolerant species (*Populus tremula*) species. We address the question whether physiological adjustments in antioxidant composition in response to growth irradiance are reversible in shade-tolerant and -intolerant species on a sudden change in light environment, and whether the potential to carry out these adjustments depends on previous growth irradiance.

**Materials and Methods**

**Study site**

The work was conducted in a natural, temperate, mixed deciduous forest in Järselja, Estonia (58°22′ N, 27°20′ E, elevation 38–40 m) in 2000. The stand is dominated by the shade-intolerant species *Populus tremula* L. and *Betula pendula* Roth. in the upper canopy layer (15–25 m), while the lower canopy layer (4–17 m) is dominated by the shade-tolerant species *Tilia cordata* Mill. The stand leaf-area index is = 6 m²·m⁻², and < 5% of above-canopy irradiance reaches the understory vegetation. A detailed site description is provided by Niinemets *et al.* (1998).

**Study design**

We started with the experimental treatments on 30 June (*T. cordata*) or 1 July (*P. tremula*), and the experiment continued until 10 July (*T. cordata*) or 11 July (*P. tremula*). Both plant species form only one leaf flush at the beginning of the season, and the leaves were fully mature at the start of the experiment. Leaves were selected from different light environments within the natural canopy light gradient to cover the full range of natural variation in foliar pigment contents, morphology and photosynthesis potentials, as described in detail by Niinemets *et al.* (2003). Overall, 16 distinct canopy positions in *P. tremula* and eight locations in *T. cordata* were chosen, and each of these leaves was illuminated individually with additional irradiance of = 500–800 μmol m⁻² s⁻¹ between 05:00 and 21:00 hours by wide-beam (beam angle 60°) 65 W halogen dichroic lamps (Decostar Titan, Osram GmbH, Munich, Germany). A very stable leaf light environment was achieved by attaching the lamps directly onto tree branches. For every leaf with extra irradiance, a representative neighbouring control leaf without extra light was also selected.

Shade bags made of neutral-density shade cloth were installed on further 16 leaves of *P. tremula* and eight leaves of *T. cordata*. The shade bags transmitted 45% of incident light. A representative control leaf was also chosen for each shaded leaf. This resulted in total numbers of control leaves of 32 for *P. tremula* and 16 for *T. cordata*.

Extra illumination increased leaf temperature on average by 1.63 ± 0.38°C, and the temperature in shaded bags was 0.33 ± 0.15°C. However, there was no height effect on the temperature difference. These data demonstrate that the experimental treatments had an effect on leaf temperature, but also that this effect did not interact with leaf growth irradiance. For a more detailed description of the experimental design see Niinemets *et al.* (2003).

**Determination of growth and extra irradiance**

Long-term irradiance during leaf development (growth irradiance) was determined by a method combining hemispherical photography and estimations of quantum flux density above the canopy. Hemispherical photographs were taken above each leaf, and the fractions of penetrating diffuse (I_D) and direct (I_B) irradiance for 15 d after the summer solstice were determined from the photographs as in Niinemets *et al.* (1998). The average daily integrated photosynthetic quantum flux density (Q_int, mol m⁻² d⁻¹) for the period 21 May–11 July was calculated as:

\[
Q_{int} = Q_{int}^0 [I_D P_D + I_B (1 - P_D)]
\]

Eqn 1

where \(Q_{int}(\text{mol m}^{-2} \text{d}^{-1})\) is the daily average integrated above-canopy quantum flux density for the period selected (36.7 mol m⁻² d⁻¹ in our study) and \(P_D\) is the fraction of diffuse light.

Daily leaf irradiance for each experimental day was determined by combining the hemispherical photoanalyses with continuous measurements of quantum flux density in 13 canopy locations, as described in detail by Niinemets *et al.* (1998). This method is based on regressions between the values of \(I_D\) and \(I_B\) and the integrated daily irradiances \([Q_d(\text{day})]\) estimated from the sensor readings:

\[
Q_d(\text{day}) = a I_D + b I_B
\]

Eqn 2

where \(a\) and \(b\) are the regression coefficients. The amount of light intercepted each day until the sampling for leaf pigments was calculated using the same approach, developing leaf- and day-specific regressions using the sensor readings integrated until leaf sampling \([Q_{ts}(\text{day})]\).

Total daily light interception \([Q_d(\text{day})]\) of leaves with extra light was calculated as \(Q_d(\text{day}) + Q_{es}(\text{day})\), where \(Q_d(\text{day})\) is the daily integrated extra irradiance. The integrated irradiance until leaf sampling for pigments \([Q_{ts}(\text{day})]\) was the sum of \(Q_{es}(\text{day})\) and the extra irradiance until the sampling \([Q_{es}(\text{day})]\). The values of \(Q_d(\text{day})\) and \(Q_{es}(\text{day})\) were further employed to calculate the cumulative total light and extra light intercepted from the start of experimental treatments until leaf sampling.

**Sampling of foliage for antioxidant analyses**

Leaves with extra illumination and corresponding control leaves were sampled every day for the first 6 d, and on the last (11th) day after the start of the treatments. Leaves with shade bags and their controls were sampled on the fifth and 11th day after the start of shading. Leaf sampling was conducted...
between 15:00 and 16:00 hours. At each sampling time two discs of area 0.22 cm$^2$ were removed using a cork-borer, placed in labelled vials, and plunged into liquid nitrogen within 5–10 s of removal. During the entire experiment more than 400 samples were collected. The samples were stored at $-80^\circ$C until analyses.

**Analytical methods**

For determination of lipophilic antioxidants and VAZ cycle pigments, leaf samples were extracted with acetone from leaf discs (0.22 cm$^2$) and analysed by reverse-phase HPLC following the method of García-Plazaola & Becerril (1999) with the modifications described by García-Plazaola & Becerril (2001). Detection was carried out with a fluorescence detector (Waters model 474) set to $\lambda_{\text{exc}} = 295$ nm and $\lambda_{\text{em}} = 340$ nm, calibrated with $\alpha$-, $\beta$-, $\delta$- and $\gamma$-tocopherol standards (Calbiochem, San Diego, CA, USA). Under these chromatographic conditions we were unable to separate $\beta$- from $\gamma$-tocopherol.

For extraction of hydrophilic antioxidants, frozen leaf samples were ground in a mortar with liquid nitrogen and extracted with HClO$_4$ (2.5 kmol m$^{-3}$) and neutralized with a succinate buffer (200 mol m$^{-3}$ pH 12.7) to reach a final pH between 4 and 5 for ascorbate determination, or between 6 and 7 for glutathione determination. In neutralized aliquots total ascorbate was measured spectrophotometrically as described by García-Plazaola et al. (1999). Total glutathione was assayed spectrophotometrically according to the method of Griffith (1980).

**Statistics**

Linear regression was used to analyse the relationships between antioxidant contents with growth irradiance ($Q_{\text{int}}$). Calculated $P$ values and regression lines are indicated on the figures whenever significant at $P < 0.05$. The effect of extra light or shading on antioxidant composition at species level was tested by covariance analysis (ANCOVA) with the corresponding untreated leaves as controls. Data were subjected to the Kolmogorov–Smirnov test in order to check whether they met the assumption of normality. When differences were insignificant ($P > 0.05$) a common regression was fitted with all data. Species were also separated by ANCOVA analyses.

We used linear regressions to fit changes in antioxidant composition vs cumulative extra irradiance. Although some leaves showed an initial lag effect in antioxidant induction, we used all data to derive the slopes of linear relations in order to allow the comparison of all data sets and to study the total response during the experimental treatment. To test for the effect of $Q_{\text{int}}$ on these slopes (interaction of acclimation with growth light environment), linear regression analysis was also used.

**Results**

**Antioxidant variations through the canopy light gradient**

In control leaves of *T. cordata* the contents of all major antioxidants (glutathione, ascorbate and $\alpha$-tocopherol) were positively correlated with growth irradiance ($Q_{\text{int}}$), when expressed on either an area basis or a chlorophyll basis (Figs 2, 3, respectively). In the case of *P. tremula* leaves ascorbate did not correlate with $Q_{\text{int}}$, while the slopes of $\alpha$-tocopherol and glutathione vs $Q_{\text{int}}$ were smaller than for *T. cordata* ($P < 0.01$). The other tocopherols also showed a distinct pattern in both species: ($\gamma + \beta$)-tocopherol did not correlate with $Q_{\text{int}}$ (but decreased when expressed per unit total chlorophyll) in *T. cordata*, but was positively related to $Q_{\text{int}}$ in *P. tremula*. $\delta$-tocopherol was present only in *T. cordata* leaves and markedly decreased with $Q_{\text{int}}$ (Fig. 3). When both species were compared at growth irradiances where both clouds of points overlap (5–

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**Fig. 2** Variation in antioxidant (ascorbate, glutathione and tocopherols) content per unit of leaf area in relation to $Q_{\text{int}}$ in control leaves of *Populus tremula* (open squares) and *Tilia cordata* (closed circles). In the lower panel, closed triangles represent the content of $\delta$-tocopherol in *T. cordata*. Symbols represent the average for all controls of the same leaf.
10 mol m⁻² d⁻¹), ascorbate, tocopherol and glutathione were remarkably similar, indicating that there are no substantial differences in antioxidant composition between these species.

Effects of extra light and shading on antioxidant content

Extra light increased daily interception twofold for the upper canopy and 10–15-fold for the lower canopy. Despite this large increase and the influence of \( Q_{\text{int}} \) on the antioxidant pools, we did not detect any significant effect of the altered light environment in the case of most hydrophilic antioxidants (Fig. 4). An exception was the significant increase of glutathione content in \( T. \text{cordata} \) leaves exposed to extra light (Fig. 4).

The content of all lipophilic antioxidants responded to extra light in both species, but shading did not lead to significant changes (Fig. 5). After 11 d of elevated leaf irradiance, the content of \( \alpha \)-tocopherol and (\( \beta + \gamma \))-tocopherol increased in \( P. \text{tremula} \), but this increase was observed only in the upper \( Tilia \) leaves. In \( T. \text{cordata} \) the slopes of \( \alpha \)-tocopherol and (\( \beta + \gamma \))-tocopherol contents vs \( Q_{\text{int}} \) were significantly higher in leaves with extra light compared with control leaves. The slopes of \( \alpha \)-tocopherol and (\( \beta + \gamma \))-tocopherol contents vs \( Q_{\text{int}} \) were not statistically different in \( P. \text{tremula} \) leaves. By contrast to these responses, the content of \( \delta \)-tocopherol (not present in \( P. \text{tremula} \)) was not affected, or decreased with extra light, in the lower-canopy leaves of \( T. \text{cordata} \).

Kinetics of tocopherol changes

Changes in tocopherols were monitored for 11 d (Fig. 6). During this time \( \alpha \)-tocopherol steadily increased in response
to extra light in all leaves except for the *T. cordata* lower canopy, where it remained stable. Despite this large increase and relatively long duration of the experiment, the increase in α-tocopherol content did not follow a saturation kinetic. The content of (β + γ)-tocopherol also increased linearly in *P. tremula* leaves, but decreased for the first 6 d in lower-canopy leaves of *T. cordata*. In these leaves the same pattern was observed for δ-tocopherol changes.

The slope of α-tocopherol vs cumulative extra light (Fig. 7) was positively associated with *Q* <sub>10</sub> in both species, but in...
T. cordata the slope of these changes was significantly larger than in P. tremula. This species difference indicates a stronger effect of growth light environment on the plastic adjustments of α-tocopherol content to changes in light conditions in T. cordata. This was also the case for (β + γ)-tocopherol, which responded more strongly to Qint in T. cordata than in P. tremula. The same pattern was observed in δ-tocopherol (absent in P. tremula). Although the slopes of responses to extra light for all tocopherols increased with Qint in T. cordata, it must be noted that the slopes were negative in the deepest shade conditions for δ-tocopherol and (β + γ)-tocopherol, and close to zero for α-tocopherol.

The total α-tocopherol and VAZ pools were also correlated in control leaves of both species (Fig. 8). The slopes of α-tocopherol vs cumulative extra light also correlated with those of VAZ vs cumulative extra light in T. cordata (Fig. 8), but not in P. tremula. This effect was because the slope of adjustment of VAZ vs cumulative extra light in P. tremula was not correlated with Qint, although the absolute values were larger than those in T. cordata.

Discussion

Canopy light gradients and antioxidant pools

The content of all major antioxidants (ascorbate, glutathione and α-tocopherol) increased with growth PPFD (30-fold variation in irradiance) within the canopy of T. cordata and P. tremula, but important differences were observed between species, with a stronger response in low-canopy T. cordata leaves. At comparable growth irradiances both species contained the same antioxidant composition, indicating that there are no species-specific differences, at least in intermediate irradiances. Despite the different ecological characteristics of both species, which could be reflected in their respective antioxidant responses, we focused our study mainly on the interaction of leaf response with leaf growth irradiance, irrespective of species. When expressed on an area basis, the antioxidant pools increased by a factor of 1.5 for glutathione, 2.6 for ascorbate, and 10 for α-tocopherol from...
bottom to top of the canopy. Despite the high variability in absolute hydrophilic antioxidant contents among different plant species (Logan et al., 1996), differences between sun and shade leaves were consistent with reported previous observations in natural light gradients, for example sun/shade ratios for ascorbate: 3.1 in Citrus aurantium (Schwanz et al., 1996); 4.8 and 3.3 in Fagus sylvatica (García-Plazaola & Becerril, 2001); 2.4 in Quercus petraea (Hansen et al., 2002); 1.9 in Quercus robur (Hansen et al., 2003); 1.9 in Quercus ilex (García-Plazaola et al., 1999); 1.8 in Vinca minor (Logan et al., 1998a), and ratios for glutathione: 1.3 in C. aurantium (Schwanz et al., 1996); 2.4 and 1.8 in F. sylvatica (García-Plazaola & Becerril, 2001; Hansen et al., 2002); 2.0 in Q. petraea (Hansen et al., 2002); 1.7 in Q. robur (Hansen et al., 2003); or 1.5 in Q. ilex (García-Plazaola et al., 1999).

By contrast to hydrophilic antioxidants, the natural variation of α-tocopherol contents with irradiance is characterized by a much higher variability. A 10-fold higher content in sun leaves relative to shade leaves has been reported for F. sylvatica (García-Plazaola & Becerril, 2001), while no growth irradiance-dependent trend has been found for V. major and Cucurbita pepo (Logan et al., 1998a). The largest variability in α-tocopherol vs irradiance relations could be explained by the fact that, irrespective of crown position, this antioxidant undergoes a progressive accumulation during leaf ageing. This effect has been observed for several temperate woody species such as F. sylvatica (García-Plazaola & Becerril, 2001), Q. robur (Hansen et al., 2003), Corylus avellana, Cornus sanguinea, Betula pubescens, P. tremula and Alnus glutinosa (García-Plazaola et al., 2003a), and probably represents a general feature in deciduous species. In sun leaves of all these species, the α-tocopherol to chlorophyll ratio reached a peak in early autumn close to, or higher than, 1. As the maximum α-tocopherol per unit of chlorophyll in thylakoid membranes is in the range of 0.02–0.04 (Hansen et al., 2003), and much of this compound accumulates in plastoglobuli (Tevini & Steinmüller, 1985), it is not clear whether all this α-tocopherol accumulation during leaf ageing in deciduous species actually contributes to photoprotection (García-Plazaola et al., 2003a; Hansen et al., 2003).

As described for several species (Grusak & DellaPenna, 1999; Munné-Bosch & Alegre, 2002b), α-tocopherol was the predominant form in leaves of both P. tremula and T. cordata, except for the lower canopy of T. cordata. By contrast to α-tocopherol, the irradiance trends of other tocopherol forms differed between the two species. In P. tremula the content of (β + γ)-tocopherols mimicked the irradiance response of α-tocopherol, while in T. cordata both (β + γ)-tocopherol and δ-tocopherol decreased with Qnet. These four tocopherol forms represent the two final branches of the tocopherol biosynthetic pathway, with α-tocopherol being the end product that results from methylation of γ-tocopherol, and β-tocopherol the end product that results from methylation of δ-tocopherol (Hofius & Sonnewald, 2003). The absence of δ-tocopherol in P. tremula could indicate that, in contrast to T. cordata, the δ-, β-branch of the tocopherol biosynthetic pathway is not present in leaves of this species. Assuming the lack of δ- and β-tocopherol in P. tremula, and considering that we were unable to separate the isomers β- and γ-tocopherol, the (β + γ)-tocopherol pool of Figs 5–7 would be composed exclusively of the γ form in P. tremula, while in T. cordata the largest proportion would correspond to the β form. This evidence, along with the data reported for Arabidopsis (Bergmüller et al., 2003) or Spinacia (Fryer, 1992), suggests that the intermediate γ-tocopherol always represents a minor proportion of the tocopherol pool in leaves. However, it has been demonstrated by the use of Arabidopsis mutants deficient in γ-tocopherol methyltransferase that α-tocopherol functions can be replaced by γ-tocopherol with no major impact on protection against photo-oxidative stress (Bergmüller et al., 2003).

In lower-canopy leaves of T. cordata α-tocopherol accounted for only 24% of the tocopherol pool. To our knowledge this is the first report showing that the α form is not the main tocopherol in photosynthetic tissues. Given that the antioxidant activity of δ-tocopherol in vivo is the lowest among tocopherols (Grusak & DellaPenna, 1999; Munné-Bosch & Alegre, 2002b), the highest proportion of δ-tocopherol in lower-canopy leaves may be the outcome of the very low light stress that the lower-canopy leaves are exposed to in natural conditions. However, the role of these nonubiquitous tocopherols is not yet fully understood.

Antioxidant changes in response to alterations in light environment

Exposure of leaves to shading or extra light did not result in any change in hydrophilic antioxidants. Minor responses to increases in growth irradiance in ascorbate pools have been described in other species (Logan et al., 1998b; Ye et al., 2000), and suggest that hydrophilic antioxidant content was probably large enough to compensate for any increases in irradiance. During light treatments, high de-epoxidation activity observed in leaves of these species in a complementary study (Niinemets et al., 2003) provides evidence that ascorbate availability, at least, is not limiting violaxanthin de-epoxidase activity (Müller-Moulé et al., 2002).

By contrast to ascorbate and glutathione, extra light resulted in dramatic changes in tocopherol contents and derivatives. At the end of the treatment α-tocopherol had increased by a factor of 1.6–2.8 in all canopy positions except for the lower-canopy leaves of T. cordata, where it remained stable (Fig. 5). Rapid changes of α-tocopherol have been described in epiphytic ferns exposed to light and desiccation (Tausz et al., 2001) and in Arabidopsis exposed to light or temperature stress (Havaux et al., 2000; Bergmüller et al., 2003). Longer-term increases of α-tocopherol have also been reported in Mediterranean shrubs in response to drought (Munné-Bosch et al., 1999) or winter stress (García-Plazaola et al., 2003b). However, other authors have shown a depletion
of α-tocopherol in leaves exposed to severe photo-oxidative stress (Wise & Naylor, 1987; Mnüne-Bosch et al., 2001). This depletion precedes chlorophyll loss, supporting the role of α-tocopherol in protecting chlorophyll from oxidation. Although it is known that α-tocopherol is the main lipophilic antioxidant to scavenge ROS directly in the lipid phase of thylakoid membranes (Fryer, 1992), the essential role of this molecule in maintaining photosynthetic activity at high light intensities has been examined only recently, by blocking tocopherol biosynthesis in Nicotiana tabacum (Grañês et al., 2001) and Chlamydomonas reinhardii (Trebst et al., 2002). Its role, as well as that of zeaxanthin, in preventing membrane photo-oxidation is discussed in a later section (Is there an interplay between α-tocopherol and zeaxanthin?).

During the course of the experiment, α-tocopherol accumulation induced by extra light proceeded linearly. We did not detect a saturation phase in the kinetics of α-tocopherol increase, suggesting that 11 d was not enough to reach a new equilibrium between the antioxidant pool size and the new light environment caused by extra illumination. Thus antioxidant pool size responds to changed irradiance with a lower rate than expected based on growth chamber experiments. A similar lack of saturation with time was reported previously for VAZ pool size increase and chlorophyll depletion for this canopy (Niinemets et al., 2003). It could be argued that the relationship between chlorophyll degradation and α-tocopherol accumulation results from the fact that α-tocopherol synthesis is fed by the products of chlorophyll degradation, as has been proposed for senescing leaves (Rise et al., 1989). However, in our experiment it is unlikely that accumulation of tocopherols and decrease in chlorophylls were coupled, as the rates of α-tocopherol synthesis were not correlated with those of chlorophyll loss. It could also be interpreted that this α-tocopherol increase is caused by accelerated ageing induced by extra light. Although we cannot exclude this possibility, it seems unlikely as chlorophyll and electron transport rate decreased only moderately (Niinemets et al., 2003), and it has been shown that accelerated ageing leads to a depletion of α-tocopherol in damaged beech leaves (García-Plazaola & Becerril, 2001).

Changes in tocopherol forms differed between both species. In P. tremula (β + γ)-tocopherol increased in parallel with α-tocopherol, caused by illumination with extra light, while in the upper leaves of T. cordata the (β + γ) form continued to increase and δ-tocopherol did not change. By contrast, there was a depletion of (β + γ)- and δ-tocopherols in the lowest leaves of T. cordata. The observed decrease in δ-tocopherol and (β + γ)-tocopherol in parallel with the maintenance of α-tocopherol in deep shade leaves of T. cordata exposed to extra light may indicate that there is an interconversion among them that maintains the initial size of the α-tocopherol pool. This suggests that δ-tocopherol and (β + γ)-tocopherol act as a reservoir for the biosynthesis of the more potent antioxidant α-tocopherol. This is the case for α-tocopherol formation from γ-tocopherol, but it is currently unclear whether conversion of δ- and β-tocopherols to γ and α forms is biochemically possible. Irrespective of the possible role of these nonubiquitous carotenoids as a source for α-tocopherol synthesis, our data clearly show that these compounds are depleted when leaves are exposed to a higher level of excitation energy, and this appears to correspond with their antioxidant capacity (Fryer, 1992).

### Shade tolerance and antioxidant defences

Previous studies have shown that the shade-intolerant species P. tremula has a higher capacity for photochemical energy utilization and for nonphotochemical energy quenching than the shade-tolerant T. cordata at comparable Q_{int} (Niinemets et al., 1998; Niinemets & Kull, 2001). Lower potential for usage and dissipation of irradiance in the shade-tolerant species suggests that a stronger protection capacity against photo-oxidative stress is required, as has been shown in mixed canopies of the shade-tolerant F. sylvatica and the shade-intolerant Q. petraea (Hansen et al., 2002). In the present study we also demonstrate that the responsiveness of antioxidant systems to sudden changes in light environment are higher in T. cordata, as shown by the higher slopes of α-tocopherol changes vs increased irradiance. However, we have previously demonstrated (Niinemets et al., 2003) that T. cordata has a limited capacity for VAZ pool adjustments, resulting in greater sensitivity to photoinhibition of electron transport systems in this species. Taken together, these results demonstrate that greater increase in α-tocopherol in T. cordata is not enough to compensate for the increases in irradiance. Alternatively, it can be considered that the greater increase in VAZ pool size in P. tremula is enough to dissipate the extra light energy safely, avoiding the requirement for a larger antioxidant pool.

Is there an interplay between α-tocopherol and zeaxanthin?

In the present work, and in a previous report (Niinemets et al., 2003), we have shown that acclimation to excess light is attained in the canopy of P. tremula and T. cordata by a continuous increase of α-tocopherol and VAZ pigments, with a concomitant decrease in total chlorophyll. In fact, the concentrations of α-tocopherol and VAZ were strongly correlated, and even the respective slopes of changes vs cumulative extra light were correlated in the shade-tolerant species T. cordata (Fig. 8). In P. tremula the slopes of VAZ vs cumulative extra light and α-tocopherol vs cumulative extra light were not related (Fig. 8), but the responsiveness of VAZ to extra light was proportionally higher than that in T. cordata. Strong correlations between zeaxanthin and α-tocopherol contents have been reported by other authors (Grañês et al., 2001). These correlations are consistent with the hypothesis that both compounds play a synergistic role in membrane fluidity and protection from photo-oxidation.
(Havaux & Niyogi, 1999). It has recently been demonstrated that the combination of zeaxanthin and α-tocopherol in liposomal systems exerted a synergistic protection against lipid peroxidation (Wrona et al., 2003). The interdependence of acclimation in α-tocopherol and VAZ pigments could be a general response in plant canopies, as has also been observed in Mediterranean ecosystems during periods of unusually low air temperatures (García-Plazaola et al., 2003b). Overall, the results of this field experiment do not probe the hypothesis of a synergistic effect between tocopherols and zeaxanthin, but provide additional evidence to support that both compounds may cooperate in preventing photo-oxidation.

Conclusions

Leaves in natural forest canopies acclimate to light gradients by an extensive set of morphological, physiological and biochemical changes. Biochemical acclimation is based on the modulation of chlorophyll, VAZ, ascorbate and α-tocopherol pools. All these metabolites are strongly interconnected in their physiological functions. Ascorbate is required for the enzymatic formation of zeaxanthin but also for regeneration of α-tocopherol from α-tocopheroyl radicals, while zeaxanthin and α-tocopherol exert a synergistic protection against lipid peroxidation. In the present study, parallel changes in α-tocopherol and VAZ were the basis of antioxidant acclimation, but leaf acclimation potential depended on the previous leaf light environment. When growing at comparable irradiances the highest capacity for α-tocopherol formation was observed in T. cordata (Fig. 7), corresponding to the intrinsic lower rates of excess energy dissipation in this shade-tolerant species. Shade-intolerant species such as P. tremula, with higher pools of VAZ, had a more limited antioxidant response to sudden changes in irradiance.

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