

# The significance of xanthophylls and tocopherols in photo-oxidative stress and photoprotection of three Canarian laurel forest tree species on a high radiation day

Agueda María González-Rodríguez<sup>1</sup> \*, Michael Tausz<sup>2</sup>, Astrid Wonisch<sup>2</sup>, María Soledad Jiménez<sup>1</sup>, Dieter Grill<sup>2</sup>, Domingo Morales<sup>1</sup>

<sup>1</sup> Departamento de Biología Vegetal, Universidad de La Laguna, E-38207 La Laguna, Tenerife, Spain

<sup>2</sup> Institut für Pflanzenphysiologie, Karl-Franzens-Universität Graz, Schubertstraße 51, A-8010 Graz, Austria

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## Summary

Chloroplast pigments, chlorophyll fluorescence, and tocopherols in sun leaves of the Canarian laurel forest species *Myrica faya*, *Laurus azorica*, and *Persea indica* were characterized during the diurnal course of a high irradiation summer day. All species showed a de-epoxidation of the xanthophyll cycle and a decline of the photochemical efficiency of photosystem II measured as the Fv/Fm fluorescence ratio. Whereas in *M. faya*, decline and recovery of Fv/Fm was only correlated to zeaxanthin formation, these relations were divergent in *L. azorica* and *P. indica*. In *P. indica*, Fv/Fm did not fully recover until the next morning. Apparently, the decrease of Fv/Fm during the day reflected only a zeaxanthin-dependent, quickly recovering component in *M. faya*, but an additional component, probably due to photodamage processes requiring repair activities, was observed in *L. azorica* and *P. indica*. In leaves of those two species,  $\alpha$ -tocopherol concentrations varied during the diurnal course, probably indicating active oxygen scavenging action. In *P. indica* leaves, a degradation and restoration of chlorophyll contents were observed during the day. The conclusion is that *M. faya* is most resistant to high light stress and *P. indica* most sensitive, which coincides with known ecological traits of these species.

**Key words:** antioxidants – *Laurus azorica* – *Myrica faya* – *Persea indica* – photochemical efficiency – photodamage

## Introduction

The impact of high irradiation on the photosynthetic organs of plants causes a potentially destructive excess of light energy that is absorbed by the chlorophylls. Plants respond with a

reversible decrease of the photochemical efficiency of photosystem (PS) II, known as photoinhibition, which can be measured as the decrease in the ratio of variable to maximal chlorophyll *a* fluorescence (Fv/Fm). The photoinhibition can be resolved in slow recovering and fast recovering components (Thiele et al. 1998). The fast recovering component (up to about 1 h) is probably related to the formation of zeaxanthin.

\* E-mail corresponding author: aglerod@ull.es

xanthin in the xanthophyll cycle (Jahns and Miede 1996). Zeaxanthin is highly effective in the heat dissipation of excess excitation energy. The reversible de-epoxidation of violaxanthin, which contains two epoxide groups, to zeaxanthin (without epoxide groups), with the intermediate mono-epoxide antheraxanthin, is called the xanthophyll cycle (Demmig-Adams and Adams 1994). The amount of excitation energy dissipated by the xanthophylls is dependent on the pool size and on the de-epoxidation state: more epoxides (violaxanthin) means less energy dissipation and less photoprotection. The slowly recovering photoinhibition is presumably related to the photodegradation and turnover of the D1 protein of the PS II reaction center (Thiele et al. 1998). It probably involves the action of reactive oxygen species (ROS), which are formed by the transfer of excess excitation energy from chlorophylls to molecular oxygen. The resulting activated singlet oxygen can lead to the formation of hydroxyl radicals, lipid peroxidation chain reactions in the thylakoid membranes, and oxidation of proteins and pigments (Elstner and Osswald 1994, Foyer and Noctor 2000). Carotenoids are potential scavengers of ROS directly in the pigment bed (Young 1991). The lipid soluble antioxidant  $\alpha$ -tocopherol is located in the thylakoid membranes, and counteracts the effects of ROS by removing oxidized substrates or stopping the lipid peroxidation chains initiated by ROS (Fryer 1992).

The capacity of plants to withstand or adapt to high light stress is an important ecological property involved in their resistance to environmental stresses (Polle 1997, Foyer and Noctor 2000). Responses of photoprotective traits to stress impacts such as summer drought or seasonal cold stress have been found repeatedly (Polle and Rennenberg 1994, Kyriacou et al. 1995, Munné-Bosch and Alegre 2000, García-Plazaola et al. 2000). Recent studies also emphasized the contribution of photoinhibition and photoprotection to competition in different forest ecosystems, such as deciduous broadleaves (Kitao et al. 2000), tropical forests (Thiele et al. 1998), and Mediterranean evergreens (García-Plazaola et al. 2000).

The Canarian laurel forest ecosystems mainly cover the very humid section of the Northern slopes in the medium elevations of the Canary Islands where cloud belts govern the water regime. Laurel forest trees are typically evergreen, sclerophyll broadleaved species of different systematic origin. In contrast to Mediterranean sclerophyllous forests, drought periods are mitigated by the high frequency of fogs in the Canarian laurel forests. The air humidity is typically very high and the forest has the appearance of a cloud forest (Hollermann 1981). Photostress situations occur mainly due to the high irradiation, up to  $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$  during sunny periods. Cloudless summer days probably represent the strongest environmental stress situation with which these trees have to cope. The differences in the photoprotective responses to such strong stress conditions can help to explain ecological differences and limitations of different species.

Among the tree species investigated in the present investigation, the Myricaceae *Myrica faya* is found in most laurel for-

est plots, but also grows well at degraded sites and outside the range of the typical laurel forest ecosystems. *Laurus azorica* (Lauraceae) is one of the most important tree species in the typical Canarian laurel forest ecosystems, but may also play a role in the succession from degraded sites to closed canopy stands. *Persea indica* (also Lauraceae) is represented in dense and closed canopy forest sites where it is highly competitive (González-Henríquez et al. 1986). In a previous study, pigments and photosystem II efficiency (Fv/Fm) of these species were measured in the morning and at mid-day on a sunny day in spring (Morales et al. 1997). The results pointed toward a relationship between protective pigments and photoinhibition and the ecological properties of the species, but a detailed analysis of photoprotective strategies was not possible with that approach. Therefore, the present study analyzes a complete diurnal course of chloroplast pigments, chlorophyll fluorescence, and tocopherols, with the objective of characterizing differences in the photoprotective strategies of these species.

## Materials and Methods

### Field site and plant materials

The investigations were carried out at an experimental site at Agua García mountain, Tenerife, Canary Islands (28° 27' 32" N and 16° 24' - 20" W). The climate at this site is humid mediterranean with average annual temperature of 14 °C, average minimum of 10.7 °C, and an average maximum of 17.7 °C, average relative humidity of 80 %, and an annual precipitation of 733 mm. The forest is situated at 820 m a.s.l. on a slight NNE orientated slope (8–12°). Meteorological data during the investigation period are presented in Figure 1.

A wooden scaffolding tower gave access to the top of the canopy. Dominant, about 37 years old and 20 m high individuals of three tree species were investigated: *Persea indica* (L.) K. Spreng., *Laurus azorica* (Seub.) Franco (both Lauraceae), and *Myrica faya* Ait. (Myricaceae). A detailed description of the forest stand structure is given in Morales et al. (1996 a, b). All measurements were made on the youngest, fully developed leaf age class of the sun canopy.

### Environmental data

Environmental data were read every 30 min using a PAR sensor (Li-Cor, Lincoln, USA) and a thermo-hygrometer positioned at the tower platform (Fig. 1). Leaf temperatures were measured at the lower leaf surfaces using a contact thermocouple (Type K, Thandar Ltd., UK).

### Sampling times

Sampling was conducted during the diurnal course of an absolutely cloudless period at the end of June. Three replicates were taken at each sampling time (see Figures).

## Relative water contents (RWC)

Relative water contents were determined on leaves collected into pre-weighed plastic bags, which were sealed. Leaf fresh mass (fw) was determined and the weight at water saturation (sw) was determined after leaving the leaves for 24 hours, immersing their petioles in water in a water-saturated atmosphere. Leaf dry mass (dw) was determined after oven drying the leaves at 100 °C for 48 h. RWC was calculated as the proportion of water content *in situ* (fw-dw) to the maximal water content at saturation (sw-dw), expressed in %.

## Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence was determined using a portable fluorimeter (Plant Efficiency Analyser PEA, Hansatech, UK). Measurements were made after the leaf part was dark-adapted for 30 min using leaf clips. The maximal apparent efficiency of PS II was calculated as Fv/Fm. The values are regarded as representative of the time when the dark adaptation clip was set.

## Pigments and tocopherols

Whole leaves were cut at the petiole and immediately (within seconds) frozen in liquid nitrogen. The leaves were lyophilized, ground in a dismembrator under liquid nitrogen and the leaf powder was stored under humidity-proof conditions at -25 °C until analysis.

Pigments were determined using the HPLC gradient-method described by Pfeifhofer (1989). This method permits separation of all important chloroplast pigments in one step: Column Spherisorb S5 ODS2 250×4.6 mm with precolumn S5 ODS2 50×4.6 mm. Solvent A: acetonitrile : methanol : water = 100 : 10 : 5 (v/v/v), solvent B: acetone : ethylacetate = 2 : 1 (v/v), linear gradient from 10 % solvent B to 70 % solvent B in 18 min, run time 30 min, flow 1 mL min<sup>-1</sup>, photometric detection at 440 nm. The leaf dry powder was shaken in ice-cold acetone and centrifuged at 2 °C and 10,000×g. The supernatants of three re-extractions were combined and adjusted to a fixed final volume. Extracts were injected (20 µL) using a cooled autosampler.

Tocopherols were determined following a method by Wildi and Lütz (1996), which was slightly modified: Acetone extracts (see above) were subjected to a isocratic HPLC analysis (Column Spherisorb S5 ODS2 250×4.6 mm with precolumn S5 ODS2 50×4.6 mm) using methanol as solvent. Tocopherols were detected directly by fluorometry (excitation 295, emission 325 nm).

## Statistical analysis

Statistical analyses were completed using the Statistica (StatSoft, USA) software package.

Variables with a coefficient of variance (CV) greater than 15 % are drawn along the diurnal course (Figs. 2–4) to show the time-dependent variations (except for  $\alpha$ -carotene, where high CVs are mainly due to the low concentrations). Data were pooled according to the species, and species-dependent differences in means were evaluated using one-way analysis of variance followed by least squared differences post-hoc comparisons (results in Table 1). Species-dependent differences in the variations during the diurnal course were evaluated using the Levene's test.  $P < 0.05$  is regarded as significant.

## Results

Ambient data during the investigation period are shown in Figure 1. Leaf temperatures amounted to between 19 °C in the morning (all species) and maximum of 29 °C (*M. faya*), 31 °C (*P. indica*), and 26 °C (*L. azorica*). Relative water contents during the daily course ranged between 92 and 84 % (maximum and minimum for *M. faya*), 96–91 % (*P. indica*), and 98–92 % (*L. azorica*), respectively.

In Table 1, average chlorophyll concentrations are presented per unit leaf dry weight. Carotenoids and tocopherols

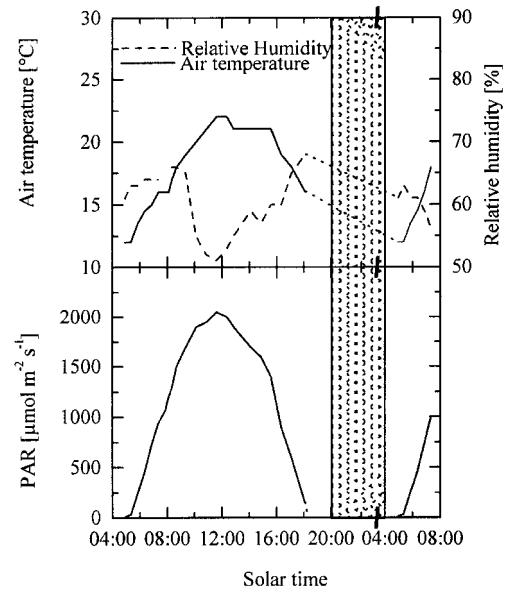


Figure 1. Ambient data during the investigation period.

Table 1. Average concentrations of pigments and tocopherols in sun leaves of three tree species of the Canarian laurel forest. Means  $\pm$  standard deviation (coefficient of variance in %) of  $n = 24$  measurements. Different lower case letters indicate significant differences of means, different capital letters significant differences in variance between species. V = violaxanthin, A = antheraxanthin, Z = zeaxanthin, dw = leaf dry weight.

	<i>Myrica faya</i>	<i>Laurus azorica</i>	<i>Persea indica</i>
Total chlorophyll [ $\mu\text{mol g}^{-1}\text{dw}$ ]	2.38 $\pm$ 0.26 a (11)A	2.59 $\pm$ 0.40 ab (15)AB	2.99 $\pm$ 0.64 b (21)B
Carotenoids and tocopherols [nmol $\mu\text{mol}^{-1}$ chl]			
V+A+Z	71 $\pm$ 4 a (5)	107 $\pm$ 9 b (8)	100 $\pm$ 9 b (9)
Neoxanthin	32 $\pm$ 2 a (5)	37 $\pm$ 2 b (6)	38 $\pm$ 4 b (10)
Lutein	158 $\pm$ 9 a (6)	154 $\pm$ 11 a (7)	189 $\pm$ 21 b (11)
$\alpha$ -Carotene	0	6 $\pm$ 1 (20)	3 $\pm$ 1 (29)
$\beta$ -Carotene	78 $\pm$ 9 ab (11)	69 $\pm$ 8 b (12)	86 $\pm$ 9 a (11)
$\alpha$ -Tocopherol	142 $\pm$ 12 a (8)A	123 $\pm$ 28 a (23)B	68 $\pm$ 18 b (27)B
$\gamma$ -Tocopherol	2 $\pm$ 1 (28)	2 $\pm$ 2 (80)	56 $\pm$ 8 (15)

are presented per unit chlorophyll to emphasize the protective potential per captured light energy.

*M. faya* leaves contained less chlorophyll per unit dry weight, less neoxanthin, and a smaller pool of the xanthophyll cycle pigments compared to *P. indica* and *L. azorica*.  $\alpha$ -Carotene was not found in samples of *M. faya*. Total tocopherol levels were comparable in the three species, but in *P. indica* leaves,  $\gamma$ -tocopherol accounted for nearly half of the total tocopherol concentrations, whereas it was below 2% in leaves of the other two species.

Diurnal variations were greatest in total chlorophyll contents, in the tocopherols, in the chlorophyll fluorescence  $F_v/F_m$ , and in the de-epoxidation state of the xanthophyll cycle (CVs in Table 1 and Figs. 2, 4, and 5). Neoxanthin, lutein, the xanthophyll cycle pool size, and  $\beta$ -carotene had smaller variations (lower coefficients of variance), and the relatively high CVs in  $\alpha$ -carotene data was mainly caused by the low contents of this pigment. These data were therefore not presented along the time course.

The xanthophyll cycle de-epoxidation state calculated as  $(Z+0.5 \cdot A)/(V+A+Z)$  changed with the photosynthetic active radiation (PAR) during the day in leaves of all three species. Pre-dawn values were about 0.2 and maximum values at midday greater than 0.8 (Fig. 2).

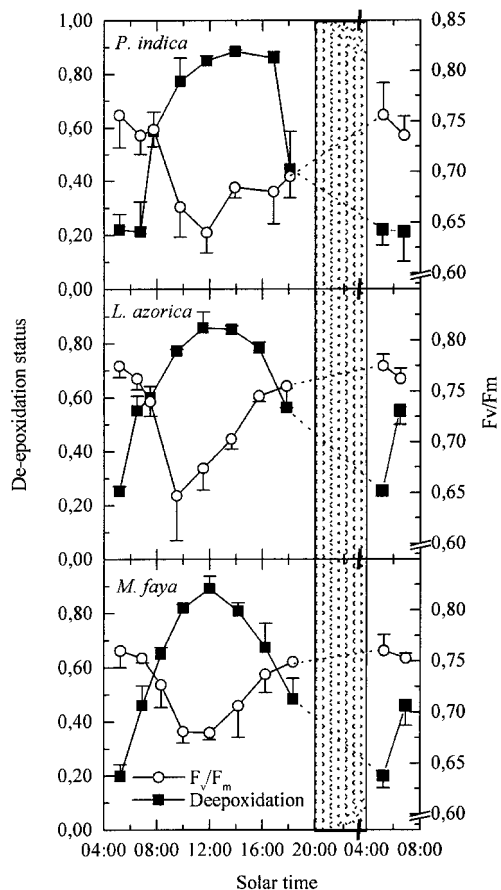
In all three species,  $F_v/F_m$  ratios decreased markedly during the first hours of the day followed by recovery during the afternoon. The amplitude of the changes was smallest in *M. faya*. In *P. indica*, foliage  $F_v/F_m$  ratio stayed lower and only fully recovered overnight, whereas in *L. azorica* and *M. faya*, pre-dawn values were already matched by evening values (Fig. 2).

Although in all species there was a temporal coincidence between the de-epoxidation of the xanthophylls and the depression in  $F_v/F_m$ , the scatterplots in Figure 3 highlight possibly important mechanistic differences. While in *M. faya* leaves the relation between  $F_v/F_m$  and the de-epoxidation state is the same in both the depression and recovery during the day (indicated by the arrows in the graphs), it is different in *L. azorica* and *P. indica*. The recovery of  $F_v/F_m$  follows a different line than the depression, with the difference in *P. indica* the being the most pronounced.

Total chlorophyll concentrations varied most in *P. indica* leaves, indicating a degradation even during the morning hours followed by a recovery in the afternoon (Fig. 4).  $\alpha$ -tocopherol concentrations varied more in *P. indica* and *L. azorica*, both exhibiting a minimum around high noon of solar time. The variations of total chlorophyll and  $\alpha$ -tocopherol were very small in *M. faya* leaves, indicating the more constant pools of these metabolites (Fig. 5).

## Discussion

Although the present experiment was carried out on a completely sunny day, the relative water contents (RWC) of the

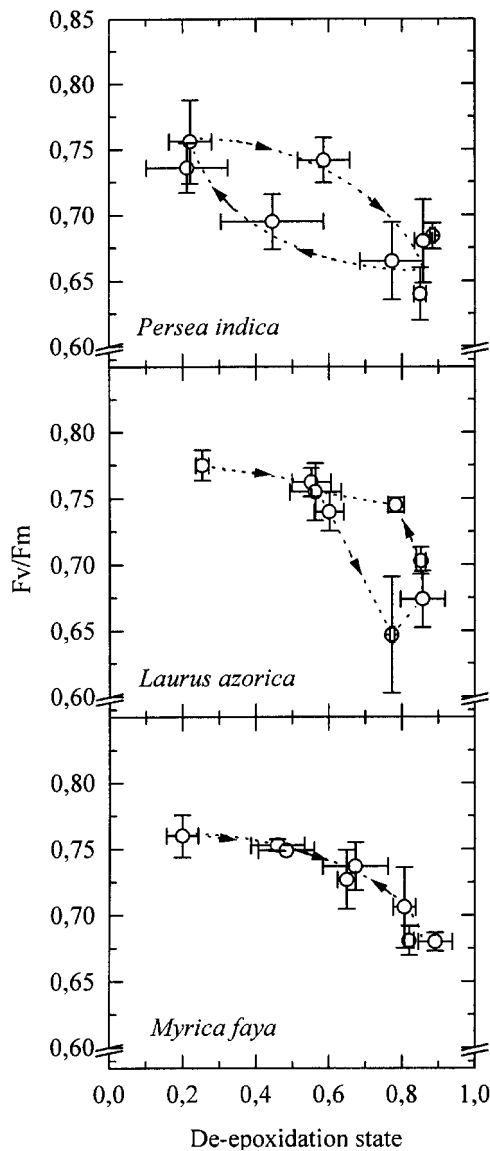


**Figure 2.** The de-epoxidation state of the xanthophyll cycle (calculated as  $(Z+0.5 \cdot A)/(V+A+Z)$ ) and  $F_v/F_m$  ratio of chlorophyll fluorescence in the sun foliage of three laurel forest tree species along the diurnal course of a cloudless day. Data are medians and half total data spans of 3 measurements each.

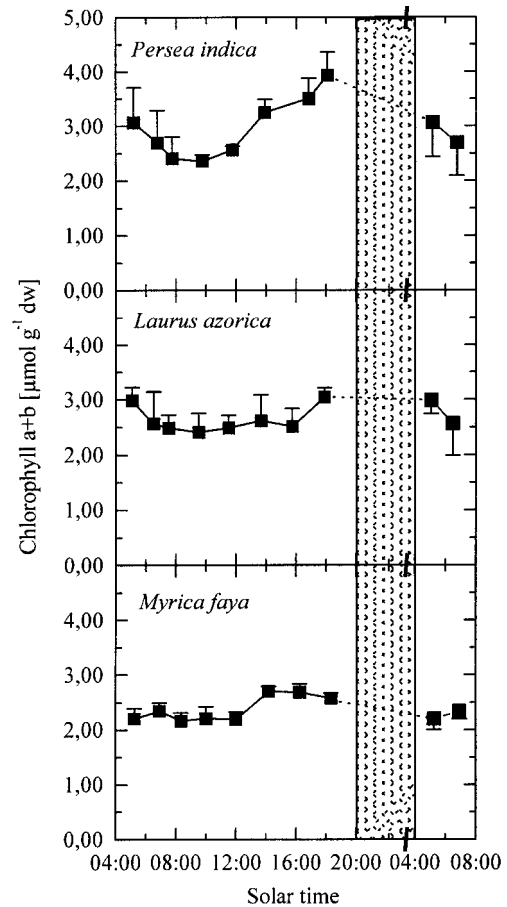
leaves were never below 80%, which indicates that dehydration was not a decisive factor. Investigations of laurel forest species (Jiménez et al. 1999) and other plants (Kaiser 1987) have suggested that dehydration affects photosynthetic apparatus only at RWCs below 70%. The main stress factor was the high irradiation causing photooxidative stress (Polle and Rennenberg 1994) and possibly also causing the rise of the leaf temperatures. The photosynthetic optimum of the three investigated species is at leaf temperatures of about 25 °C (González-Rodríguez 1998). Higher leaf temperatures lead to a decrease in  $\text{CO}_2$  fixation and, hence, further increase the possibility of photooxidative stress.

Species-dependent differences have repeatedly been observed in pigment composition (Thayer and Björkman 1990, Morales et al. 1997). Sun-adapted species or individuals generally contain less chlorophyll per g leaf dry weight and a higher xanthophyll cycle pool per unit chlorophyll (Thayer and Björkman 1990). From this point of view, the differences among the species do not allow a clear conclusion to be

drawn about their ecological character. *M. faya* had the lowest concentrations of chlorophyll, which would indicate a more sun-adapted character, and *P. indica* had the highest, which would point towards a more shade-adapted foliage. On the other hand, *M. faya* had the smallest amount of the xanthophyll cycle pigments per unit chlorophyll, which is a characteristic usually ascribed to more shade-adapted plants (Thayer and Björkman 1990), and *P. indica* generally contained more carotenoids per unit chlorophyll, which was previously assumed as indicative of sun-adapted plants. The direct comparison of these characteristics among different species is difficult, and numerous dynamics may exist along



**Figure 3.** The de-epoxidation state of the xanthophyll cycle plotted against the Fv/Fm ratio of chlorophyll fluorescence. The lines and arrows indicate the changes along the time course of the day. Data are medians and total spans of 3 measurements each.

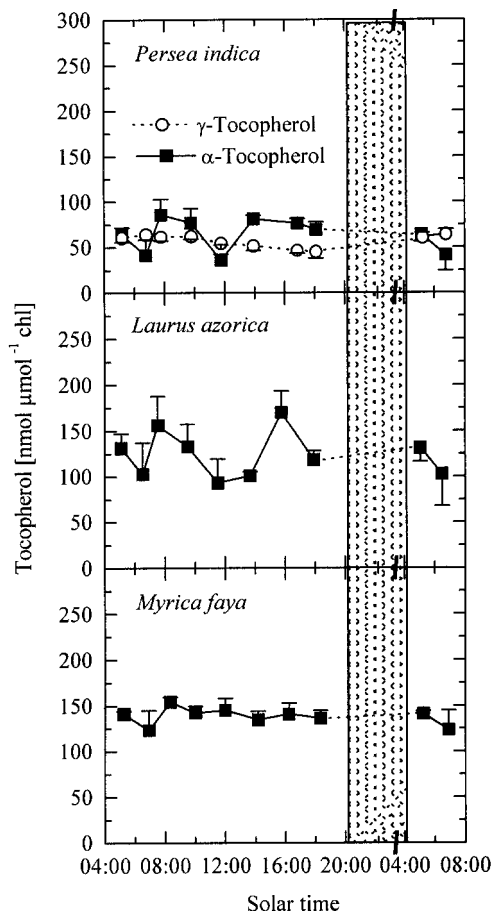


**Figure 4.** Total chlorophyll concentrations in the sun foliage of three laurel forest tree species along the diurnal course of a cloudless day. Data are medians and half total data spans of 3 measurements each.

the seasonal course (García-Plazaola et al. 1997, compare also the data in Morales et al. 1996 c, 1997). Since only sun leaves were taken into account in the present investigation, the comparisons between sun/shade stress adaptations may be of limited value, and the diurnal dynamics of photoprotective pigments and photoinhibition *in situ* may be of more ecological relevance.

The depression of the photochemical efficiency of photosystem II, measured as Fv/Fm, was observed in numerous studies in many plants under high light impact (photoinhibition). This depression has been ascribed to multiple mechanisms, among them fast recovering ones, such as the formation of zeaxanthin in the xanthophyll cycle, and slowly reversible ones involving structural changes in the thylakoid membranes or degradation of proteins in the photosystems requiring *de-novo* synthesis (Thiele et al. 1998).

The xanthophyll cycle responses of the investigated species were very similar and closely followed the daily course of the illumination, but in the Fv/Fm response there were substantial differences. Only in leaves of *M. faya*, did the depression and recovery of Fv/Fm directly follow the de-epoxidation



**Figure 5.** The tocopherol system in the sun foliage of three laurel forest tree species along the diurnal course of a cloudless day. Data are medians and half total data spans of 3 measurements each.  $\gamma$ -Tocopherol in *M. faya* and *L. azorica* leaves was low (Table 1) and is not shown.

state of the xanthophylls, whereas in *P. indica* and *L. azorica* a sharp decrease of Fv/Fm in the first hours was followed by a beginning recovery before high noon while the xanthophyll de-epoxidation state continued to increase (Fig. 2). In contrast to *M. faya* and *L. azorica*, the Fv/Fm ratios in *P. indica* leaves were still lower in the evening than they were the following morning. The data plotted in Figure 3 suggest that there are distinct mechanisms involved in the induction of the Fv/Fm decrease in *L. azorica* and *P. indica*. In *M. faya* the decline in photochemical efficiency may be exclusively connected to zeaxanthin formation (= de-epoxidation) and the recovery only to the zeaxanthin epoxidation, as indicated by the congruence of the two lines in Figure 3. In *L. azorica* and *P. indica*, the decline of Fv/Fm also followed the pace of zeaxanthin formation, but the recovery showed a different time course (Fig. 3). This probably reflects a slowly reversible component of photoinhibition accounting for part of the Fv/Fm decline, for example as caused by the degradation processes of proteins by excess light energy, or by structural membrane changes caused by ROS formed in the overex-

cited photosynthetic apparatus (Jahns and Miede 1996, Thiele et al. 1998). The repair of these structures is time-consuming, and the amount of time necessary for repair is different for *P. indica* and *L. azorica*. While in *L. azorica* leaves the repair process apparently initiates a recovery of the Fv/Fm decline during the day, this process is delayed in *P. indica* and takes overnight (Figs. 2 and 3). Brugnoli et al. (1998) also reported different curvilinear regression lines for non-photochemical fluorescence quenching (which is connected to Fv/Fm) and the xanthophyll de-epoxidation state in different species and leaf types, and ascribed this to the different mechanisms involved in photoinhibition.

The hypothesis that the destructive action of ROS causes degradation processes is corroborated by the high variation in chlorophyll contents in *P. indica* during the day which even indicates a photodegradation of chlorophyll during the first hours, and a *de-novo* synthesis afterwards (Fig. 4).  $\alpha$ -Tocopherol, as the most important lipophilic antioxidant in thylakoid membranes, is responsible for the stabilization of membrane structures under conditions of oxidative stress: It acts as a chain-breaking reactant in lipid peroxidation cascades and is an effective scavenger of ROS directly in the lipid phase (Fryer 1992). A decline of the  $\alpha$ -tocopherol pool during the day has been observed in Mediterranean plants under drought stress (Munné-Bosch and Alegre 2000). In the present study, the variations were smaller, but both *L. azorica* and *P. indica* leaves showed a decrease in tocopherol contents around midday when irradiation was highest. In *M. faya*, no degradation of  $\alpha$ -tocopherol was observed during the day. *L. azorica* and *P. indica* also showed a decrease in tocopherol contents during the first morning hours. Depressions in antioxidants immediately after sunrise were also found by García-Plazaola et al. (1999) and were interpreted by them as a marker for photo-oxidative stress. In our data-set, in addition to the midday depressions of  $\alpha$ -tocopherol, early morning variations in tocopherol concentrations may indicate effects similar to those described in García-Plazaola et al. (1999).

The occurrence of high levels of  $\gamma$ -tocopherols in leaf tissues of *P. indica* is an exceptional trait of this species as compared to others (Polle and Rennenberg 1994). Since  $\gamma$ -tocopherol is thought to be the precursor in  $\alpha$ -tocopherol biosynthesis (Fryer 1992), this could indicate a high turnover rate of tocopherols in the leaves of this species, possibly compensating for the lower constitutive  $\alpha$ -tocopherol concentrations.

Higher variations of the chlorophyll concentrations and of the tocopherol pools during the diurnal course both point to an increased action of ROS within the thylakoid membranes, in other words, a stress situation that was not successfully avoided by light-dissipating mechanisms (the xanthophyll cycle). This is only observed when, as in *P. indica* and *L. azorica*, a slowly recovering decline of Fv/Fm is also present (Fig. 3). Under that condition, the effects of photostress may occur in a sequence during the day. First, a fast-recovering

photoinhibition, which is exclusively caused by the reversible zeaxanthin formation, is observed. This is the only effect of high light observed on *M. faya* on this day. Second, the slowly recovering component of photoinhibition occurs, which is independent of zeaxanthin formation, if energy dissipation through the xanthophyll cycle mechanism, and possibly other zeaxanthin-related effects (Jahns and Miehe 1996) are insufficient. Such effects are indicated by the divergent lines for inhibition and recovery in Figure 3. This type of photoinhibition probably involves the action of ROS directly in the thylakoid membranes (Jahns and Miehe 1996, Thiele et al. 1998), which are counteracted by the presence of tocopherols (Fryer 1992, Munné-Bosch and Alegre 2000). During the most stressful periods the  $\alpha$ -tocopherol pool may decrease transiently, as indicated by the significantly higher variations in the tocopherol data for *P. indica* and *L. azorica*. The possibility of this effect is supported by reports of much more pronounced degradation of  $\alpha$ -tocopherol in drought-stressed Mediterranean plants during the day (Munné-Bosch and Alegre 2000). Thirdly, if the antioxidant protection of the membranes is partly overcome by ROS, photodegradation of chlorophyll may be observed, as suggested in Figure 4 for *P. indica*. In this case, the necessary repair processes consume more time and the complete recovery takes longer.

A ranking of the three investigated species according to the sensitivity of their thylakoid membranes to photostress on a clear high irradiation day results in *M. faya* as the most resistant and *P. indica* as the most sensitive. These data fit well in the known ecology of these species within the laurel forest ecosystem dynamics. *M. faya* is able to grow in open conditions and at degraded sites. In this function its distribution is wider, also comprising fewer cloudy and more sun exposed sites where the typical laurel forest cannot fully develop. This results in photosynthetic organs with a high resistance against high light. Secondly, *L. azorica* is also able to participate in the succession leading toward a closed canopy forest, but is also competitive in the closed forest ecosystem, which, eventually, is the ecological optimum for *P. indica*.

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