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Singlet oxygen scavenging by leaf flavonoids contributes to sunlight acclimation in *Tilia platyphyllos*

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Abstract

Both high photosynthetically active radiation (PAR) and ultraviolet radiation (UV) are capable of causing photooxidative stress, but leaves are equipped with an array of protective mechanisms making life under full sunlight possible. Comparing acclimation strategies of *Tilia platyphyllos* leaves we found that sun leaves were better protected against stress than shade leaves by having (i) more efficient regulated non-photochemical quenching (ii) a higher capacity to neutralize singlet oxygen, a reactive oxygen species known to be capable of promoting oxidative damage by excess PAR and (iii) containing more UV absorbing pigments. HPLC-MS^n analysis showed both quantitative and qualitative differences in higher flavonoid contents: Sun leaves contained 4.2-times more flavonoids than shade leaves and the quercetin:kaempferol ratio was also higher in the former. In addition, sun leaves also contained significant amounts of myricetin, which was detectable only in traces in shade leaves. Flavonols were mainly present as rhamnosides and *in vitro* tests of these compounds showed that quercetin and myricetin glycosides were much better singlet oxygen antioxidants than kaempferol glycosides. Thus a shift from monohydroxylated flavonols (kaempferol derivatives) towards dihydroxylated quercetin or trihydroxylated myricetin improves the singlet oxygen targeted antioxidant potential of leaves and potentially improves protection against photoinhibition by high PAR. On the other hand, experiments with pure test compounds also showed that multihydroxylated flavonol rhamnosides do not have better UV-B absorption than monohydroxylated ones. Thus the observed difference in flavonoid composition is not expected to contribute to the observed better UV-B absorbing capacity of sun leaves. Our data suggest that responses to high intensity PAR and to solar UV-B are closely onnected and that UV-inducible flavonols play a key role in the successful acclimation of sun leaves to high PAR as efficient singlet oxygen antioxidants.

Key words

singlet oxygen; sunlight acclimation; UV; flavonoid; quercetin; kaempferol; myricetin

1. Introduction

Plants grown in a natural environment are exposed to variable light conditions both in terms of photosynthetically active radiation (PAR, 400-700 nm) and solar ultraviolet (UV, 290-400 nm) radiation. Light conditions which individual plant leaves experience vary

significantly depending on weather conditions, position, canopy shading, etc. Reflecting long-term acclimative adaptation mechanisms, leaves grown in shaded areas of a plant differ from the sun grown leaves in morphology, physiology, chemical composition and anatomy (Boardman, 1977; Lichtenthaler et al. 1981; Hallik et al., 2012). Photoprotective mechanism are important constituents of light acclimation and high solar radiation tolerance includes both physical and biochemical defenses (Niyogi, 1999; Takahashi et al. 2011). The former include leaf and chloroplast movements, epidermal screening (Solovchenko and Merzlyak, 2008) and dissipation of excess energy (Müller et al. 2001). Chemical defense pathways equip the leaf for situations when physical mechanisms fail to protect the photosynthetic electron transport system from over-excitation and, potentially from photoinhibition (Long et al. 1994; Powles, 1984) via the formation of damaging reactive oxygen species (ROS). During photoinhibition by excess PAR singlet oxygen ($^1O_2$) is photoproduced by the reaction centre chlorophyll of Photosystem (PS) II (Macpherson et al. 1993; Hideg et al. 1994). Shorter wavelength (higher energy) solar UV-B radiation (290-315 nm) is also a potential inducer of oxidative stress (Jansen et al. 1998). Laboratory experiments with high UV doses showed the presence of both $^1O_2$ and superoxide anion radicals ($O_2^{\bullet-}$) in irradiated spinach leaves (Barta et al. 2004), although the damage initiated by $^1O_2$ and derived reactive compounds was more characteristic to visible light induced photoinhibition (Hideg et al., 2000, Barta et al., 2004). While biochemical defence against $O_2^{\bullet-}$ is mainly achieved by a specific enzyme, superoxide dismutase, which is included in the supportive water-water cycle (Asada, 1999a), damage by $^1O_2$ can only be controlled by non-enzymatic antioxidants. Singlet oxygen quenching plant antioxidants include lipid-soluble compounds, e.g. β-carotene, α-tocopherol, plastoquinones, and water-soluble molecules, such as ascorbate, vitamin B6 and flavonoids (Triantaphylides and Havaux, 2009).

Flavonoids are secondary plant metabolites within the group of phenolics and are present in plants mainly as glycosides (see Hernandez et al. 2009 for a recent review). Transcriptome analyses of UV-B photoreceptor (UV RESISTANCE LOCUS8 (UVR8; Oravecz et al. 2006) mutants exposed to low fluence rates of UV-B indoors have shown that UVR8 is required for the induction of genes with important functions in UV protection including flavonoid synthesis pathways (Brown et al. 2005; Favory et al., 2009). Field studies of silver birch leaves have shown that besides UV-B, solar UV-A also regulates gene expression and the accumulation of flavonoids (Morales et al. 2010) and a recent comparative study of genes encoding flavonoid biosynthesis proteins in sun exposed wild type and uvr8

mutant Arabidopsis leaves suggested an interaction of UVR8 with other photoreceptors to modulate UV-A responses in the presence of UV-B (Morales et al. 2013).

Antioxidant properties of flavonoids have been extensively studied *in vitro* due to their nutritional and medical relevance (Rice-Evans et al. 1995, 1996, Pietta 2000; Scalbert et al. 2005). Flavonoids in leaves are typically regarded as UV-B regulated compounds, because higher UV-B radiation leads to an enhanced biosynthesis and accumulation through the induction of the phenylpropanoid pathway (Jansen et al. 2008; Kim et al. 2008; Zhang and Björn, 2009). Increased leaf flavonoid production in response to high solar irradiances or UV-B radiation has primarily been considered as physical defence mechanism, due to the UV screening function of these compounds, but flavonoids are also known as antioxidants (Harborne 1986).

Whether the primary role of flavonoids in UV defence is direct screening or radical scavenging is a controversial issue and the general view of flavonoids acting predominantly as radiation shielding screen has been challenged by experimental evidence of their strong antioxidant activities and presence in mesophyll layers and chloroplasts (for reviews see Hernandez et al. 2009; Winkel-Shirley 2002, Agati and Tattini 2010; Agati et al. 2012, Brunetti et al. 2013). Flavonoids are synthesized by a multi-enzyme complex located on the cytoplasmic surface of the endoplasmic reticulum and are transported to the vacuolar compartment or to the cell wall (Winkel-Shirley 2002). However, flavonoids were also found in the chloroplasts of many vascular plants (Saunders and McClure 1976) and recently their *in situ* synthesis was also proved in isolated kidney bean chloroplasts (Zaprometov and Nicolaeva, 2003).

In addition to good total antioxidant capacities which have been the focus of nutritional studies for decades (Rice-Evans et al. 1996, Sekher Pannala et al. 2001, Brunetti et al. 2013, Chen and Chen 2013), flavonoids were shown to be good $^1O_2$ antioxidants *in vitro* (Tournaire, 1993) as well as in leaves of a Mediterranean shrub, *Phillyrea latifolia* (Agati et al. 2007). In plants $^1O_2$ is primarily associated with damage by excess PAR and the well-documented inducibility of flavonoids by UV suggests a link between responses to these two spectrally distinct environmental factors. The aim of the present study was to test the hypothesis whether ambient solar UV radiation would promote defence against photoinhibition *in vivo*, via enhancing flavonoid synthesis. We studied large leaved linden (*Tilia platyphyllos*) leaves from sun exposed and shaded areas of the same tree. Sun and shade leaves have already been studied extensively for decades but the novel aspect of our work is a comparative study of their $^1O_2$ antioxidant capacities and UV absorbing

characteristics including both leaf extracts and pure test compounds representing flavonoids in which the two leaf types differ mostly. Members of the *Tilia* genus have high flavonoid content not only in flowers but in leaves and bracts as well (Toker et al. 2001) making this species a good model for the present study.

2. Materials and Methods

2.1. Plant material

Large-leaved linden (*Tilia platyphyllos* Scop.) leaves were collected in Szeged, Hungary (46.2460 N, 20.1673 E) in July from sun exposed and predominantly shaded parts of the same tree (referred to as sun leaves and shade leaves). The leaves of the two groups were carefully selected to avoid variance originating from size, age or other factors. On cloudless days, typical photon flux densities reaching sunny and shaded areas at noon were 1700-1800 and 80-100 µmol m^{-2} s^{-1} PAR, respectively, as measured with a Cole Parmer radiometer (Cole-Parmer Instrument Co. Ltd., London, UK). Typical ambient daily doses of biologically effective UV-B radiation on the Northern hemisphere (at N 46º) in summer are around 7.5 kJ m^{-2} d^{-1} (Bassman et al. 2001).

Chlorophyll fluorescence measurements were carried out on detached leaves following a 30 min dark adaptation period (see below). These measurements were followed by freeze-drying of the leaves (Christ Alpha 2-4 LD Freeze dryer, SciQuip Ltd, UK) at -85^{o}C and 0.060 mbar and dry weight (DW) determination. Freeze dried samples were kept at -80^{o}C until use.

2.2. Photochemical yield and non-photochemical quenching measurements

Photochemical yield and non-photochemical quenching were determined by chlorophyll *a* fluorescence measurements of sun and shade leaves using the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). Before starting the measurements, detached *Tilia platyphyllos* leaves were kept in darkness for 30 min. Of these dark adapted samples, minimum and maximum fluorescence yields (F_o and F_m, respectively) were determined before and after a saturating pulse, respectively. Following this, F and F_m' yields were obtained in a similar way at the end of short (30 sec) blue actinic light irradiations of stepwise increased intensity (Schreiber 2004). The quantum yields of PS II photochemistry, $Y(II) = (F_m' - F) / F_m'$), non-regulated dissimilative processes, $Y(NO) = F / F_m$ and regulated energy dissipation, $Y(NPQ) = F / F_m' – Y(NO)$, characteristic to each illumination step, were calculated according to Klughammer and Schreiber (2008). As these quantum yields

represent the partitioning of absorbed energy, Y(II) + Y(NO) + Y(NPQ) = 1 at each radiation wavelength.

2.3. Chemicals

Myricetin, dihydroquercetin, kaempferol quercetin-3-glucoside, kaempferol-3-glucoside, quercetin-3-rhamnoside and myricetin-3-rhamnoside standards were purchased from Carl Roth GmbH, (Karlsruhe, Germany). Kaempferol-3,7-dirhamnoside was purchcased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

2.4. Flavonol aglycon and flavonol glycoside content determination

For the flavonol aglycones 0.5 g of lyophilised leaf sample was hydrolysed with 50 % aqueous methanol and 1.6 M HCl in double determination (Krumbein et al. 2007). After refluxing at 90 °C for 2 hours, the extract was cooled down to room temperature, adjusted to 100 mL and sonicated for 5 min. The extract was then filtered through a 0.45 µm PTFE filter for HPLC analysis.

The flavonol glycosides were prepared using a modified method following Schütz et al. (2004). For this a lyophilised leaf sample (0.5 g) was extracted with 15 mL of 60 % aqueous methanol on a magnetic stirrer plate for 1.5 hours in double determination. The extract was filtered through a fluted filter and subsequently evaporated to dryness. The residue was dissolved in 5 mL of distilled water and then filtered through a CME filter (cellulose mixed ether-membrane filter) for the HPLC analysis.

An HPLC series 1100 from Agilent (Waldbronn, Germany) consisting of a degaser, binary pump, autosampler, column oven and photodiode array detector was used to quantify the flavonol aglycones and flavonoid glycosides. For identification purposes, an ion trap mass spectrometer (Agilent series 1100 MSD) was used with ESI as an ion source in negative ionisation mode. Nitrogen was the dry gas (12 l/min, 350 °C) and nebulizer gas (40 psi). Helium was the inert collision gas in the ion trap. Details of the analysis of flavonol aglycones and flavonol glycosides are given below.

The flavonol aglycones were quantified after acid hydrolysis using a modified HPLC-DAD-ESI-MS^n method (Krumbein et al. 2007). The extracts were separated on a Prodigy (ODS 3, 150 x 3.0 mm, 5 µm, 100 Å) column (Phenomenex, Aschaffenburg, Germany) with a security guard C18 (ODS 3, 4 x 3.0 mm, 5 µm, 100 Å) at a temperature of 25 °C using a water/acetonitrile gradient. Solvent A consisted of 99.5 % water and 0.5 % acetic acid; solvent B contained 100 % acetonitrile. The following gradient was used for eluent B: 30-

35 % (0-5 min), 35-39 % (5-17 min), 39-90 % (17-21 min), 90 % isocratic (21-26 min), 90-30 % (26-29 min), 30 % isocratic (29-34 min). Flow was performed using 0.3 mL min^{-1}, and the measured detector wavelength was 370 nm. The standards dihydroquercetin, kaempferol and myricetin were used to obtain an external calibration curve in the range of 0.1 to 10 mg 100 mL^{-1}. The total flavonol concentration was calculated as the sum of the concentration of the individual flavonol aglycones quercetin, kaempferol and isorhamnetin. Quercetin, kaempferol and myricetin were identified as deprotonated molecular ions and characteristic mass fragment ions by HPLC-DAD-ESI-MS^2. The mass optimisation was performed for quercetin $[M-H]^-$ *m/z* 301.

The flavonol glycosides were analysed using the same column and eluent composition as that for aglycone determination. The following gradient was used for eluent B (100 % acetonitrile) at a temperature of 30 °C: 5-7 % (0-12 min), 7-9 % (12-25 min), 9-12 % (25-45 min), 12-15 % (45-100 min), 15 % isocratic (100-150 min), 15-50 % (150-155 min), 50 % isocratic (155-165 min), 50-5 % (165-170 min), 5 % isocratic (170-175 min) (Schmidt et al. 2010). The flow was performed using 0.4 mL min^{-1}, and the measured detector wavelength for the quantification was set at 370 nm for non-acylated flavonol glycosides and 330 nm for acylated flavonol glycosides. The standards quercetin-3-glucoside, kaempferol-3-glucoside, quercetin-3-rhamnoside, myricetin-3-rhamnoside and kaempferol-3,7-dirhamnoside were used to obtain an external calibration curve in the range of 0.1 to 10 mg 100 mL^{-1}. Mass optimisation for the ion optics of the mass spectrometer was performed for quercetin *m/z* 301. In addition, arbitrary *m/z* 1000 was used as the target mass in auto mode to include higher mass fragments. The MS^n experiments were performed in auto or manual mode up to MS^4 in a scan from *m/z* 200 to 2000. Mass fragments used for identification are described in Schmidt et al. (2010).

2.5. Singlet oxygen scavenging capacity measurements

Freeze dried samples were extracted using MeOH : H_2O (in 1 : 1 ratio) then centrifuged (6,000 x *g* for 10 min at 4 °C) and supernatants were used for measurements. Methanol extractions were used because these provided higher extraction yields than water only (Hideg and Majer, 2010) and made comparisons between ROS scavenging capacity measurements and flavonoid analysis possible.

Measurements were based on the leaf extract's ability to scavenge $^1O_2$ and therefore prevent the oxidation of 1,3-diphenylisobenzofuran (DPBF) dye (Young et al. 1973). The reaction mixture contained 20 µM methylene blue dye as $^1O_2$ source and 100 µm DPBF in 30%

methanol. DPBF oxidation was followed by a decrease of absorption at 410 nm using a Shimadzu (UV-1601) spectrophotometer. The $^1O_2$ scavenging capacity of leaf extracts and test compounds were compared to that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and expressed as µM Trolox equivalents mg^{-1} DW and µM Trolox equivalents μM^{-1} flavonoid, respectively.

2.6. Absorbance spectra measurements

Ground freeze dried samples of sun and shade *T. platyphyllos* leaves were extracted into acidified methanol (MeOH : H_2O : HCl in 79 : 20 : 1 ratio), vortexed and centrifuged (10,000 x *g*, 10 min, 4 °C) and clear supernatants (1 mg DW mL^{-1}) were used for measuring absorbance spectra (Mirecki and Teramura, 1984) with a Shimadzu UV-1601 spectrophotometer. Pure flavonoids were extracted into methanol and the solutions were diluted to 20 µg mL^{-1}

2.7. Statistical analysis

For photosynthesis and singlet oxygen antioxidant measurements five leaves were collected from the sun exposed and five from shaded parts of the same tree. Antioxidant capacity measurements of pure flavonol compounds were repeated three times. Results are given in tables or in figures as means ± standard deviations. The significance of differences between data sets was assessed using unpaired Students *t*-tests and significantly different data sets ($p<0.05$) are labelled with asterisks. Data were analysed with GraphPad QuickCalcs (GraphPad Software Inc., La Jolla, CA, USA). Graphs were created with SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

3.1. Photosynthetic response to solar radiation: sun leaves have more efficient energy dissipation than shade leaves

The partitioning of absorbed radiation energy in leaves provides information on physical photoprotective capacities. Abilities of sun and shade *Tilia platyphyllos* leaves to tolerate high solar irradiances were characterized by measuring ratios of energy used in photochemistry and dissipated in non-photochemical quenching according to Klughammer and Schreiber (2008). Ratios of photochemical yield Y(II) (circles in Fig.1A) and non-photochemical yields Y(NPQ)+Y(NO) (squares in Fig.1A) show that at irradiances higher than 80 µmol m^{-2} s^{-1} PAR sun leaves had higher photochemical yields and put relatively less

quanta into alternative dissipation pathways than shade leaves. At relatively low PAR (80 µmol m^{-2} s^{-1}) sun and shade leaves dissipated similar ratios of absorbed energy in non-regulated processes (Fig.1B). Sun leaves acclimated to higher PAR, for example to 510 or 1050 µmol m^{-2} s^{-1}, by maintaning the same Y(NO) as under low PAR. Under high PAR, which is the natural environment of these leaves Y(II) decreased to nearly zero at the expense of Y(NPQ) and all absorbed energy was dissipated (see upper row in Fig.1B). On the other hand, when shade leaves were subjected to high PAR, Y(NPQ) pathways were not capable of balancing the decrease in Y(II) and non regulated quenching increased (lower row in Fig.1B). The same tendency was observed by Hallik et al. (2012) in *Tilia cordata* leaves grown at different PAR, where shade leaves had higher values of Y(NO) but lower values of Y(II) compared to leaves grown at higher irradiance levels.

Biochemically, Y(NPQ) is realized through regulated reactions such as dissipating absorbed energy in the light harvesting complex (Demmig-Adams and Adams, 1996*a*, 1996*b*) and energy transfer from the reaction centre chlorophyll to zeaxanthin (Müller et al., 2001). High Y(NO) levels may indicate photodamage (Klughammer and Schreiber, 2008). In their natural growth site, shade leaves are sometimes exposed to high irradiances (e.g. sudden sun flecks), so in this case these fluorescence parameters measured after exposure to high PAR reflect their capacity to tolerate rare fluctuations in radiation. On the other hand, sun leaves are typically grown in high irradiance radiation conditions and therefore have acclimated to these conditions. In our study, *T. platyphyllos* sun leaves acclimated to high light with efficient physical defence pathways to avoid oxidative damage due to over-excitation. Hallik et al. (2011) proposed that as high light radiation grown *T. cordata* leaves have a higher capacity for photochemistry, this could provide them with a protection against photodamage, while shade leaves having high values of Y(NO) are in a greater danger from radiation caused stress effects. Szőllősi et al. (2010) concluded the same for the sun leaves of another tree species *Quercus petraea*, showing that these leaves were able to prevent photodamage by having higher Y(II) and Y(NPQ). We studied the physiological need of sun and shade *T. platyphyllos* leaves for additional protection by analyzing their flavonoid content. This was followed by a comparison of UV absorbing and specific $^1O_2$ antioxidant capacities of sun and shade leaf extracts and pure flavonoid test compounds identified as leaf components.

3.2. Flavonoid response to solar radiation: quantitative and qualitative differences between sun and shade leaves

After aglyconation of flavonoids, three flavonols were identified by HPLC-DAD-ESI-MS/MS: myricetin, quercetin and kaempferol (Table 1). In sun leaves the total flavonoid content (21.5 mg g^{-1} DW) was 4.2-times higher than in shade leaves (5.1 mg g^{1} DW), and significantly higher concentrations were detected of quercetin and myricetin (Table 1). In sun leaves, quercetin was the most abundant flavonol, followed by kaempferol and myricetin. In shade leaves the quantity of kaempferol exceeded that of quercetin, and myricetin could be detected only in trace amounts. The difference in quercetin concentrations was the most notable distinction between the two leaf types: sun leaves contained 22-times more of this flavonol. Toker et al. (2001) reported nine different flavonoid compounds in leaf extracts of large-leaved linden (three of them unidentified), which were various kaempferol and quercetin glycosides. Among these, quercetin compounds were more abundant, representing ca. 62% of the flavonoid content, which is very similar to the proportion of this flavonol found among the aglycones in our sun leaf samples (70%) (Table 1). To our best knowledge, ours is the first observation of myricetin in *Tilia platyphyllos* leaves.

A high quercetin to kaempferol ratio in response to either UV-B radiation or sunlight has already been reported in a large variety of plants including petunia, Arabidopsis, white mustard, white clover and bilberry (Ryan et al. 1998, 2001; Reifenrath and Müller, 2007; Hofmann et al. 2000; Jaakola et al. 2004). A common response to UV light is a more pronounced increase in quercetin, while the amount of kaempferol increases to a smaller extent or remains unaffected (Winter and Rostás 2008; Reifenrath and Müller 2007; Hofmann et al. 2000). In agreement with these published data we detected similar amounts of kaempferol in *Tilia platyphyllos* sun and shade leaves and 22-times more quercetin in sun leaves than in shade leaves (Table 1).

In addition to overall ratios of flavonols detected in acidified leaf samples, their naturally occurring glycosides were identified using HPLC-DAD-ESI-MS^n (Table 2). We found that the most prominent flavonoid glycosides in *Tilia platyphyllos* leaves were present as rhamnosides (Tables S1 and S2). Shade leaves were dominated by two kaempferol glycosides, kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside and kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside, which made-up approximately 70% of total flavonoid content (Table S1). This result is in agreement with the identification of kaempferol as the main aglycone in acidified extracts from shade leaves (Table 1). Also matching the aglycone pattern, kaempferol was less dominant in sun leaves which contained mainly myricetin and quercetin glycosides (Table 2). Eleven compounds were identified in extracts, but most were present in trace amounts only. The quercetin-3-*O*-rhamnoside-7-*O*-rhamnoside made-up approximately

18% of glycosylated flavonols in sun exposed leaves (Table S2). This flavonol glycoside has already been reported to be the main flavonoid in *T. platyphyllos* leaves (Toker et al. 2001) although the dependance on light conditions during growth was not analysed. In our leaf samples kaempferol glycosides were partly acylated with coumaric acid. Some quercetin glycosides were acylated with caffeic acid (Tables S1 and S2). Tiliroside, a courmaric acid acylated kaempferol glycoside was also identified by Aguirre-Hernández et al. (2010) in flowers and bracts of different cultivars of *Tilia americana* var. *mexicana*.

3.3. Sun leaves have more and better singlet oxygen neutralizing antioxidant flavonols than shade leaves

Singlet oxygen scavenging capacities of sun and shade *Tilia platyphyllos* leaves were measured *in vitro*, using a photometric assay. As shown in Fig.2A, methanol-water extracts from sun leaves had a 5-fold higher capacity to quench $^1O_2$ than extracts from shade leaves. To estimate contributions of flavonols to $^1O_2$ antioxidant capacities of sun and shade leaves, we tested pure flavonols using the same methodology. Figure 2B shows that flavonoid aglycones were stronger scavengers of $^1O_2$ than Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which is a vitamin E derivative frequently used for quantifying antioxidant capacities due to its strong reactivity to $^1O_2$ (Jung and Min 2009). Among the three studied flavonol aglycones, myricetin was the strongest $^1O_2$ antioxidant while quercetin had less scavenging capacity and kaempferol was the weakest.

The general structure of major flavonols detected in *Tilia platyphyllos* leaves is shown in Fig.3. The top row demonstrates that basic structures differ only in the number of hydroxyl groups on the B-ring: kaempferol having one hydroxyl group at position 4', quercetin having two groups at positions 3' and 4' and myricetin having three groups at the 3', 4' and 5'-positions. According to earlier studies on structure-dependent antioxidant activity relations of flavonoids, hydroxyl groups located on the B-ring enhance reactivity to superoxide radicals (Hu et al. 1995) or to a synthetic free radical (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid, ABTS) used in assessing total antioxidant capacities (Heim et al. 2002). Tournaire et al. (1993) studied flavonoid aglycone reactivities to $^1O_2$ *in vitro* and found a similar importance of B-ring hydroxyl groups for physical quenching. In accordance, we found that myricetin which contains three of these hydroxyl groups but was not included in the above study had the highest reactivity to $^1O_2$. In the study of Tournaire et al. (1993) quercetin, with a catechol structure on ring B had almost 4-times higher reactivity to $^1O_2$ was than kaempferol lacking this structure. Our measurements gave a smaller ratio, 1.3 (Fig.2B)

which could be due to differences in the applied solvents (methanol and deuterated methanol, respectively). In addition, our photometric method measures a decrease in $^1O_2$ concentration and thus it is more likely to detect only the chemical quenching of this ROS via oxidation, while Tournaire et al. determined total reactivities including both chemical and physical quenching.

The importance of a hydroxyl group activating the double bond of the C-ring in flavonols in chemical $^1O_2$ quenching was established by Tournaire et al. (1993) and it is supported by a strong decrease in $^1O_2$ neutralizing capacity when the hydroxyl group is glycosilated with a sugar moiety in flavonol glycosides. In general, glycosilated forms are weaker antioxidants than their respective aglycons (Ratty and Das 1988; Montoro et al. 2005). Differences in $^1O_2$ antioxidant capacities are shown in Fig.2B where flavonol aglycones are compared to their corresponding glycosides. Flavonol rhamnosides were chosen due to the abundance of this sugar moiety in large leaved linden flavonoids. In addition to confirming the lower $^1O_2$ quenching of glycosides compared to corresponding aglycones, as already reported for quercetin and quercetin-rutinoside (Tournaire et al. 1993), the aim of these measurements was to test whether the observed advantage of quercetin and myricetin over kaempferol is maintained when these flavonols react with $^1O_2$ as glycosylated forms. This was certainly the case for rhamnosides, although the myricetin > quercetin > kaempferol order of $^1O_2$ antioxidant capacities changed to quercetin-rhamnoside > myricetin-rhamnoside > kaempferol-dirhamnoside (Fig.2B). Because kaempferol-3-rhamnoside as matching pair for quercetin-3-rhamnoside was not available as pure test compound, two other corresponding glycosides, quercetin- and kaempferol-3-glucoside were also compared. These data showed that regardless of the nature of glycosylation compounds containing the dihydroxy-flavonol are better $^1O_2$ quenchers than mono-hydroxy-flavonols. This is a new finding in respect of specific $^1O_2$ neutralizing and it is in agreement with Zietz et al. (2010) reporting a higher total antioxidant capacity of quercetin glycosides than their corresponding kaempferol glycosides. We observed several differences between flavonoid compositions of sun and shade leaves which contribute to the higher $^1O_2$ neutralizing capacity of the former (Fig. 2A). These include (1) the presence of myricetin-glycosides (9%) in sun leaves as opposed to shade leaves (2) the increase in the ratio of quercetin to kaempferol glycosides from 0.01 in shaded leaves to 0.9 in sun leaves and (3) the larger variety of myricetin and quercetin glycosides detected in the sun leaves than in shade leaves (Table 2).

3.4. Higher amounts of flavonols in sun leaves do not provide better UV-B screening

In addition to the need of higher antioxidant capacities, higher amounts of flavonoids in sun leaves than in shade leaves can also be interpreted as physical defence against the UV region of the spectrum, i.e. screening harmful radiation by absorption (Caldwell et al., 1983; Burchard et al. 2000). To see whether the observed higher concentrations of flavonols serve this purpose in sun *T. platyphyllos* leaves, we compared UV absorbing properties of leaf extracts and of pure compounds. Figure 4A shows that extracts from sun leaves and shade leaves were different in their UV absorbing properties. These were total leaf extracts, containing both the epidermis which is best known to be responsible for UV screening (Robberecht and Caldwell, 1983) and mesophyll layers which were also reported to increase their flavonoid content in response to high solar radiation (Tattini et al. 2005).

Figure 4B illustrates that kaempferol, quercetin and myricetin aglycones absorb UV light mainly in the 350-400 nm spectral region, with slightly different wavelength maxima. Maximum absorption of quercetin-3-glucoside and kaempferol-3-glucoside was shifted by approximately 10 nm towards shorter wavelengths compared to their corresponding aglycones (Fig 4C). Absorption maxima of the studied flavonol rhamnosides were between 345-355 nm with slight differences in peaks (Fig.4D). Differential spectra in Figs.4E and 4F show that a selective increase in quercetin over kaempferol improves UV screening in the UV-A (315-400 nm) but not in the UV-B (290-315 nm) region of the solar spectrum. Therefore, higher amounts and larger variety of myricetin and quercetin glycosides than kaempferol glycosides cannot be accounted for the observed better UV-B absorption of sun leaves as compared to shade leaves. This result suggests that the increase in quercetin to kaempferol glycoside ratios observed in response to UV radiation (Ryan et al. 1998, 2001; Reifenrath and Müller 2007; Hofmann et al. 2000) or sunlight (Jaakola et al. 2004) is unlikely to serve solely the purpose of UV-B screening.

3.5. Hypothesis: solar UV facilitates the acclimation of sun leaves to high PAR via promoting $^1O_2$ antioxidant flavonols

In summary, comparing flavonol contents of sun and shade *T. platyphyllos* leaves we found a more pronounced difference in quercetin and myricetin glycosides than in kaempferol glycosides. A general increase in flavonoids is likely to improve UV screening but the above change in flavonols cannot be interpreted as the main constituent of photoprotection against potentially damaging UV-B. Other phenolic compounds such as hydroxycinnamic acid derivatives would serve specific UV-B screening functions much

better (Harborne and Williams 2000) while multihydoxylated flavonols promote acclimation to sunlight by other means. To support a role different from direct UV absorption, Götz et al. (2010) showed that the action spectrum of quercetin biosynthesis induction in Arabidopsis leaves did not match the absorption spectrum characteristic to flavonols, but quasi-linearly increased with higher energy radiation.

We found that the observed changes in flavonol glycoside composition gave better antioxidant defence against $^1O_2$ to *T. platyphyllos* sun leaves as compared to shade leaves. Improved protection against reactive oxygen species has been established as defence against potential oxidative damage by high PAR or UV-B radiation (for reviews see Mittler 2002; Apel and Hirt 2004; Asada, 1999b). However, $^1O_2$ is unique among pro-oxidants associated with light stress and thus accumulation of effective $^1O_2$ antioxidants may signify more specific plant responses. In earlier laboratory experiments we detected $^1O_2$ when tobacco and spinach leaves were subject to excess PAR or high doses of UV-A but not when when stress brought about by UV-B only (Hideg et al. 2000, Barta et al. 2004).

Although ambient solar UV-B alone is not damaging to plants at middle latitudes of the Northern hemisphere, it may enhance the effect of other environmental factors and contribute to a synergistic stress (Hideg et al. 2013). In this picture, flavonols in sun leaves are important for the successful avoidance of photoinhibition as effective $^1O_2$ antioxidants rather than physical UV-B screeners. Morales et al. (2013) recently found that solar UV-A elicited some of the same metabolic responses as solar UV-B in Arabidopsis leaves and predicted an interaction of UVR8 with other photoreceptors to modulate UV-A responses in the presence of UV-B. Our data support this concept and also suggest another link between responses to various spectral components of sunlight. We hypothesise that in addition to the above link between responses to UV-A and to UV-B, responses to high intensity PAR and to UV are also connected and that solar UV-induced changes in leaf flavonol profile promote acclimation to high PAR by helping to prevent $^1O_2$ mediated oxidative stress. Molecular mechanisms behind this connection are subject to further studies.

4. Supplementary data

Table S1: Fragmentation pattern of flavonol glycosides and other phenolic compounds present in shaded *Tilia platyphyllos* leaves resulting from HPLC-DAD-ESI-MS^n measurements.

Table S2: Fragmentation pattern of flavonol glycosides and other phenolic compounds present in sun exposed *Tilia platyphyllos* leaves resulting from HPLC-DAD-ESI-MSn measurements.

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Figure legends

Figure 1

Photosystem II quantum yield components of sun (empty symbols) and shade (full symbols) *Tilia platyphyllos* leaves. (A): light response curves of photochemistry (circles) and a sum of non-photochemical quenching components (squares). (B): Energy partitioning between photochemistry (black segments, Y(II)), regulated (dark gray, Y(NPQ)) and non-regulated (light gray, Y(NO)) non-photochemical quenching at three different PAR (photosynthetically active radiation) level in sun (upper row) and shade (lower row) leaves.

Figure 2

Singlet oxygen ($^1O_2$) neutralizing antioxidant capacities of (A) sun and shade *Tilia platyphyllos* leaf extracts and (B) various flavonol glycosides (standards). Error bars represent standard deviations and significantly different values ($p < 0.05$) are marked with asterisks or with different letters in figures A and B, respectively. n=5 (A) and n=3 (B).

Figure 3

Structure of major flavonol aglycones and flavonol glucosides and flavonol rhamnosides detected in *Tilia platyphyllos* leaves.

Figure 4

Absorbance spectra (A) of Tilia leaf extracts normalized to the maximum absorption of the sun leaf extract and (B-D) of pure flavonol compounds normalized to the absorption maximum of quercetin. (E-F) Differential spectra calculated from data in figures (A-D).

Figure 1

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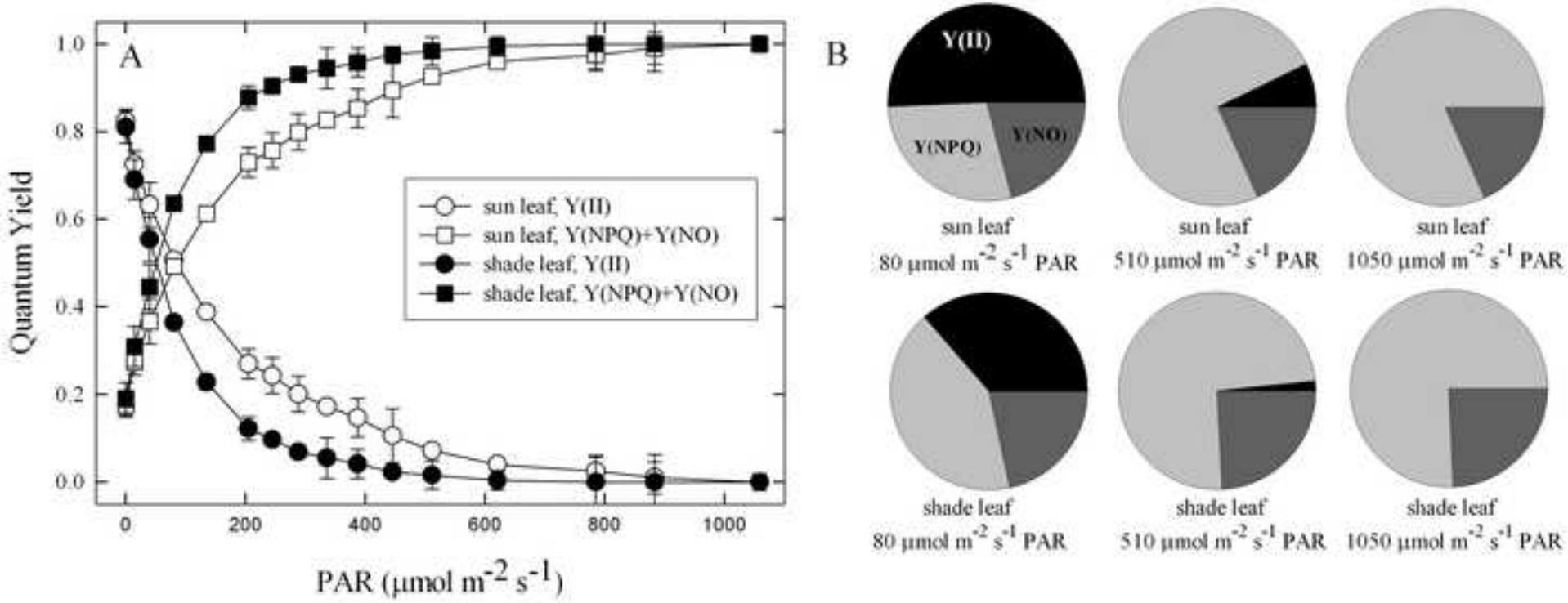

Figure 2

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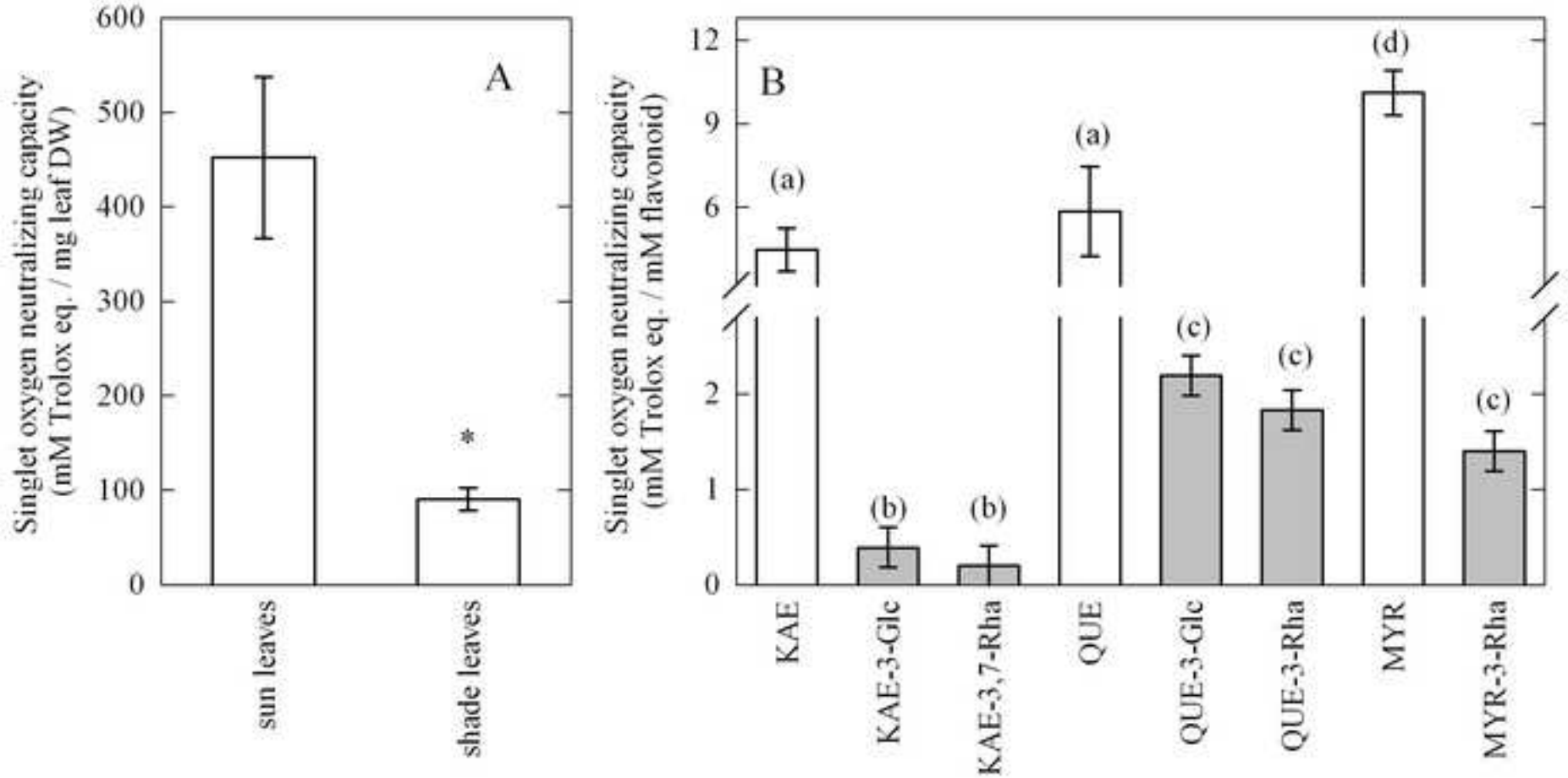

Figure 3

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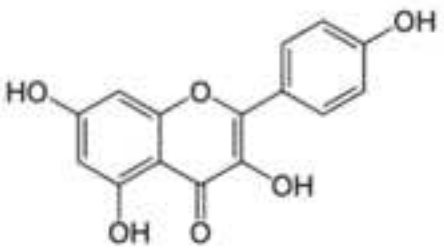
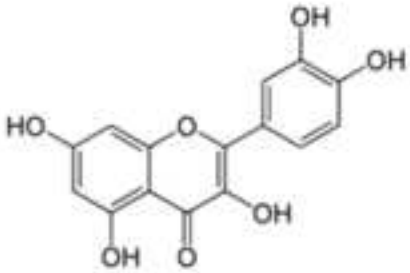
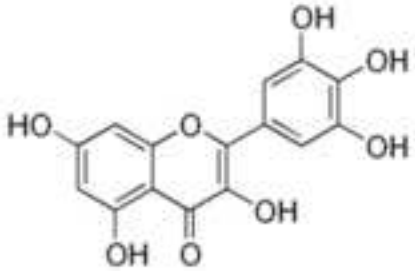
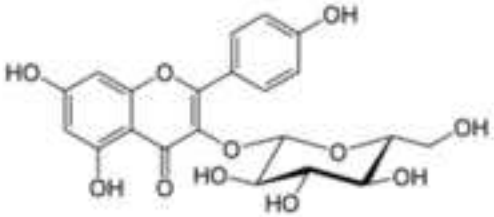
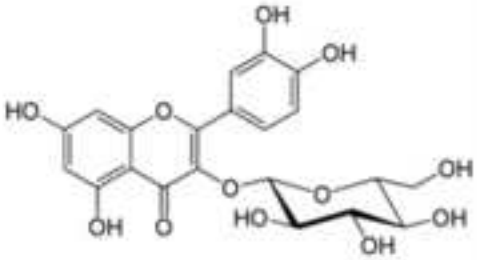
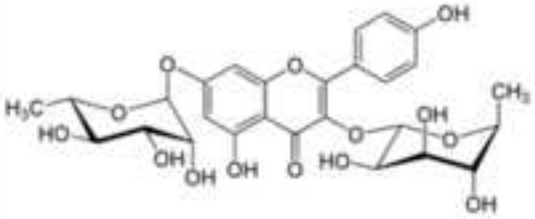
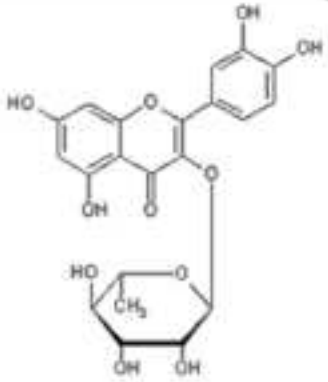
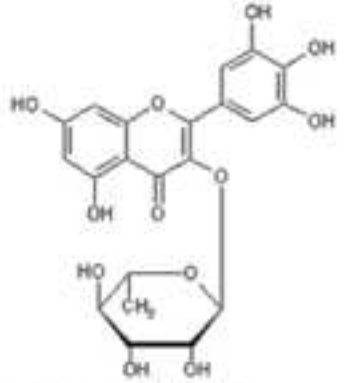
Flavonol	 KAEMPFEROL	 QUERCETIN	 MYRICETIN
Flavonol glucoside	 KAEMPFEROL-3-O-glucoside (astragalin)	 QUERCETIN-3-O-glucoside (isoquercitrin)	
Flavonol rhamnoside	 KAEMPFEROL-3,7-dirhamnoside (kaempferitrin)	 QUERCETIN-3-O-rhamnoside (quercitrin)	 MYRICETIN-3-O-rhamnoside (myricitrin)

Figure 4

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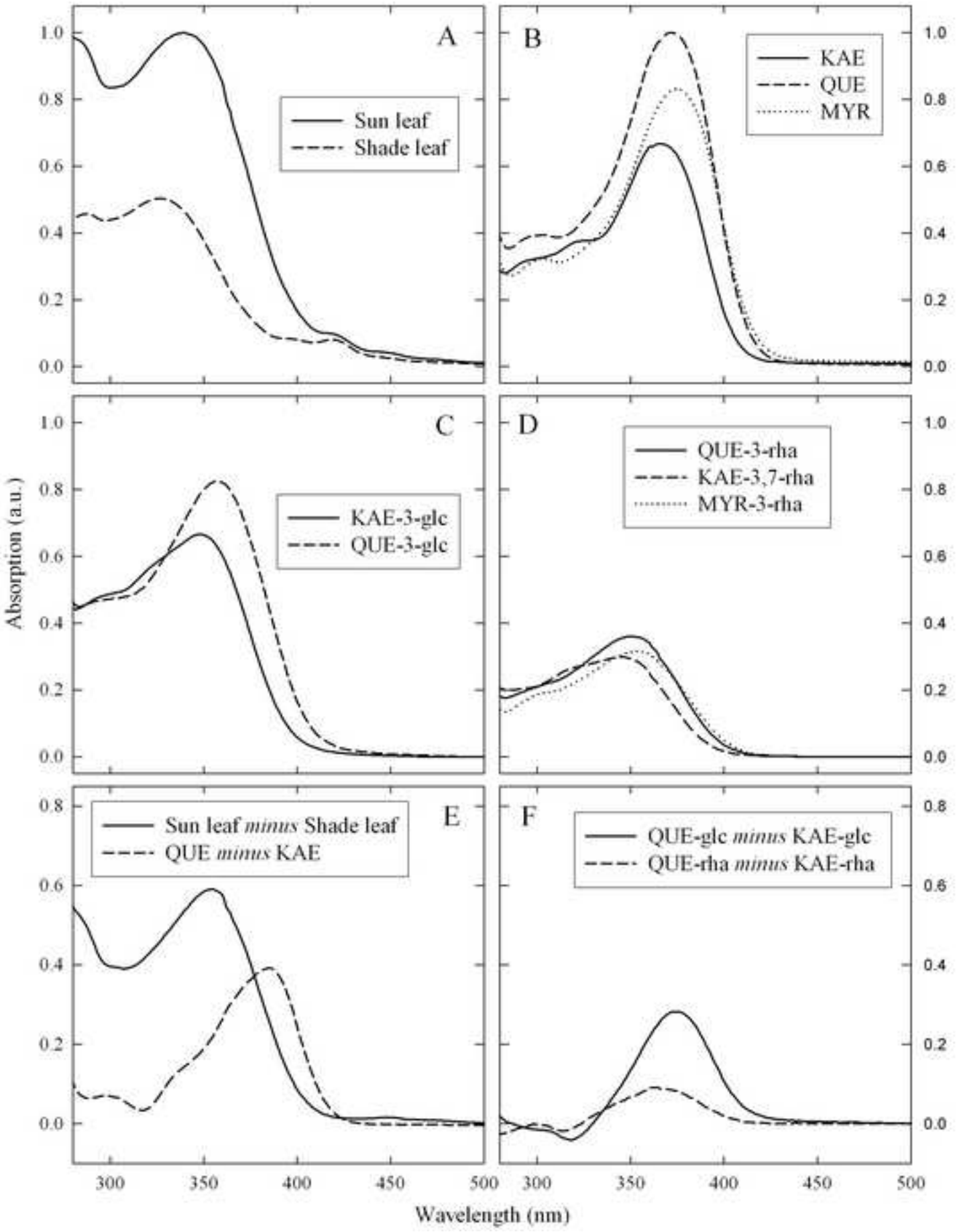

Tables

Table 1

Flavonoid aglycones of *Tilia platyphyllos* sun and shade leaves

flavonoid (mg g^{-1} dry weight)	**sun leaf**	**shade leaf**
total content	21.5 ± 5.9	5.1 ± 1.3 *
quercetin	15.2 ± 4.6	0.7 ± 0.3 *
kaempferol	4.7 ± 0.7	4.3 ± 1.0
myricetin	1.6 ± 0.6	0.1 ± 0.03 *

Table 2

Percentage composition of flavonoid glycosides and other phenolic compounds detected in *Tilia platyphyllos* sun and shade leaves

compounds	**sun leaves**	**shade leaves**
quercetin glycosides	39%	0.5%
kaempferol glycosides	42%	77.5%
myricetin glycosides	9%	traces
chlorogenic acid	3%	6%
unidentified phenol	1%	5%
unidentified compounds	6%	11%

See Table S1(shaded leaves of Tilia) and S2 (sun exposed leaves of Tilia) in Supplementary material for a more detailed list of compounds including HPLC-MS data for their identification.

Supplemental file

Singlet oxygen scavenging by leaf flavonoids contributes to sunlight acclimation in *Tilia platyphyllos*

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Table S1: Fragmentation pattern of flavonol glycosides and other phenolic compounds present in shaded linden leaves resulting from HPLC-DAD-ESI-MS^n measurements.

Shaded leaves MW	MS	MS^2	MS^3	RT (min)	λ_{max} (nm)	Compound name	Peak area in %
354	**707**, 191 (33), 353 (13)	**353**	**191**, 161 (2), 135 (1)	20.1	240, 303sh, 333	Chlorogenic acid	5
354	**707**,353 (77)	**353**	**173**,179 (57)	23.7	228, 304sh, 330	Chlorogenic acid	1
?	**591**	**295**, 179 (24), 255 (9)	179 (81), **133**, 115 (14)	34.1	241, 300sh, 331	Unknown phenol	5
594	**593**, 675 (95)	447(56), 431 (65), **285**		48.9	240, 274sh, 313	Kaempferol-3-*O*-rutinoside	0.5
610	**609**, 575 (62)	463 (52), **447**, 301 (47)	**301**	50.8	270, 296, 353	Quercetin-3-*O*-caffeoyl-7-*O*-rhamnoside	0.5
756	**755**	**593**, 447 (58), 285 (27)	**285**	63.9	230, 270, 348	Kaempferol-3-*O*-rutinoside-7-*O*-glucoside	2
594	**593**	**447**, 431 (68), 285 (36)	**284**	64.6	230, 270, 350	Kaempferol-3-*O*-glucoside-7-*O*-rhamnoside	1
564	**563**	430 (47), **417**, 285 (18)	**284**	66.4	237, 265, 351	Kaempferol-3-*O*-coumaroyl-pentoside	3
740	**739**	**593**, 431 (22), 285 (23)	**285**	84.3	268, 320sh, 346	Kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside	39
578	**577**	**431**, 285 (29)	**285**	87.1	270, 322sh, 349	Kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside	32

MW = Molecular weight, RT = Retention time, λ_{max} = Absorption maximum in nm, sh = shoulder
The numbers in brackets indicate the relative intensity of the fragment ions. Bold fragment ions conform 100 percent.

Further quercetin and kaempferol glycosides in traces (<0.5%).

Table S2: Fragmentation pattern of flavonol glycosides and other phenolic compounds present in sun exposed linden leaves resulting from HPLC-DAD-ESI-MS^n measurements.

Sun leaves MW	MS	MS^2	MS^3	RT (min)	λ_{max} (nm)	Compound name	Peak area in %
354	**707**	**353**	**191**, 179 (3), 135 (2)	19.7	247, 303sh, 334	Chlorogenic acid	2
354	**707**, 353 (91)	**353**		20.7	244, 301sh, 328	Chlorogenic aicd	1
?	**591**	**477**, 359 (14), 295 (27)	**359**, 271 (55), 315 (5)	33.8	243, 331	Unknown phenol	1
596	**595**	**462**	**315**, 179 (2)	46.8	228, 347	Myricetin-3-*O*-rhamnoside-7-*O*-pentoside	1
610	**609**	463 (76), **447**, 301 (29)	**301**	49.0	271, 357	Quercetin-3-*O*-caffeoyl-7-*O*-rhamnoside (isomer)	5
610	**609**	463 (8), **447**, 301 (33)	**301**	51.9	260, 355	Quercetin-3-*O*-caffeoyl-7-*O*-rhamnoside (isomer)	2
580	**579**	**447**, 433 (58), 301 (15)	**301**	54.7	259, 349	Quercetin-3-*O*-rhamnoside-7-*O*-pentoside	5
594	**593**	**447**, 285 (5)	**285**, 327 (45)	57.6	258, 358	Kaempferol-3-*O*-glucoside-7-*O*-rhamnoside	1
450	**449**	**316**		58.1	273, 352	Myricetin-3-*O*-pentoside	2
464	**463**	**316**		60.0	268, 360	Myricetin-3-*O*-rhamnoside	6
564	**563**	431 (55), **417**, 285 (13)	**284**	63.3	268, 355	Kaempferol-3-*O*-coumaroyl-pentoside	7
594	**593**	**447**, 431 (57), 285 (25)	**284**	64.6	268, 350	Kaemperol-3-*O*-coumaroyl-glucoside (tiliroside)	3
594	**593**	**447**, 301 (49)	**301**	65.4	269, 352	Quercetin-3-*O*-rhamnoside-7-*O*-rhamnoside	18
726	**725**	579 (75), **447**, 301 (52)	**301**	68.3	267, 353	Quercetin-3-*O*-rhamnoside-7-*O*-rhamnoside-pentoside	3
434	**433**	**301**		76.5	260, 353	Quercetin-3-O-pentoside	6
740	**739**	**593**, 431 (23), 285 (28)	**285**	83.5	270, 321sh, 349	Kaempferol-3-*O*-rutinoside-7-*O*-rhamnoside	13
710	**709**, 577 (91)	**563**, 431 (37), 285 (25)	**285**	86.5	260, 349	Kaempferol-3-*O*-rhamnoside-pentoside-7-*O*-rhamnoside	4

Sun leaves MW continued	MS	MS^2	MS^3	RT (min)	λ_{max} (nm)	Compound name	Peak area in %
578	**577**	**431**, 285 (28)	**285**	87.4	269, 353	Kaempferol-3-*O*-rhamnoside-7-O-rhamnoside	13
432	**431**	**285**		118.8	270, 322sh, 353	Kaempferol-3-*O*-rhamnoside	1

MW = Molecular weight, RT = Retention time, λ_{max} = Absorption maximum in nm, sh = shoulder
The numbers in brackets indicate the relative intensity of the fragment ions. Bold fragment ions conform 100 percent.

Further myricetin, quercetin and kaempferol glycosides in traces (< 1%).