Plant Physiology and Biochemistry, accepted manuscript

Changes in grapevine leaf phenolic profiles during the day are temperature rather than irradiance driven

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Abbreviations

ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); air T, air temperature; CA, caftaric acid; FC, total antioxidant capacity measured with the Folin-Ciocalteu method; FRAP, ferric reducing potential; g-stomata, stomatal conductance to CO_2; intern CO_2, internal sub-stomatal CO_2 concentration; KAE, kaempferol; KAE-glc, kaempferol-3-*O*-glucoside; KAE-glu, kaempferol-3-*O*-glucuronide; leaf T, leaf temperature; PAR, photosynthetically active radiation; phot CO_2, photosynthetic CO_2 uptake; QUE, quercetin; QUE-gal, quercetin-3-*O*-galactoside; QUE-glc, quercetin-3-*O*-glucoside; QUE-glu, quercetin-3-*O*-glucuronide; QUE-rut, quercetin-3-*O*-rutinoside; RH, relative air humidity; ROS, reactive oxygen species; sol UVA, solar UV-A radiation 315–400 nm; sol UVA+B, solar UV radiation 280–400 nm; sol UVB, solar UV-B radiation 280–315 nm; TEAC, Trolox equivalent antioxidant capacity; tot Flav, total flavonoid contents; tot Phen, total phenolic content; TPTZ, tripyridyl triazine; transp H_2O, water vapour transpiration; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UV, ultraviolet; WUE_i, intrinsic water-use efficiency calculated as photosynthetic CO_2 uptake divided by stomatal conductance.

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Abstract

Photosynthesis parameters, adaxial flavonoid index, phenolic profiles and antioxidant capacities of south-facing sun exposed grapevine leaves (*Vitis vinifera*, Pinot Noir cultivar) were measured hourly between 7 am and 7 pm on a clear summer day. Changes in these parameters were statistically compared to changes in environmental conditions, including solar irradiance (photosynthetically active and UV radiations), leaf and air temperature, and relative air humidity. Epidermal UV absorbance, characterized by the flavonoid index, and total extractable phenolic contents were correlated to distinct environmental parameters. The former was positively correlated to irradiance and leaf temperature, while the latter was positively correlated to air temperature. HPLC phenolic profiling identified a positive correlation between air temperature and amounts of the dominant flavonol component, quercetin-3-*O*-glucuronide. The only phenolic component statistically connected to the flavonoid index was quercetin-3-*O*-glucoside. This correlation was positive and both parameters decreased during the day, although changes in the amount of this flavonol component showed no correlation to environmental factors. Total antioxidant capacities of leaf extracts were positively correlated to solar UV, and leaf and air temperature, but not to photosynthetically active radiation. Positive correlations of quercetin-3-*O*-glucoside contents with the flavonoid index, with photosynthesis and with sub-stomatal CO_2 concentration suggest a special protective role of this flavonol. A short-term negative effect of solar UV-A and UV-B on photosynthetic CO_2 uptake was also identified, which was unrelated to changes in stomatal conductance. A hypothesis is presented assuming UV- and photorespiration-derived hydrogen peroxide as the driver of daily changes in leaf antioxidant capacities.

Key words

Vitis vinifera; solar irradiance; ultraviolet; temperature; HPLC phenolic profile; photosynthesis

1. Introduction

Ultraviolet (UV) radiation (280–400 nm) has a much larger impact on terrestrial life than its relatively small, approximately 6 % (Frederick et al., 1989) contribution to global solar radiation (Aphalo et al., 2012). Although the high energy UV component (UV-B, 280–315 nm) is also a potential stressor (Ballaré, 2003), solar UV is an important regulator of plant

growth and development (Jansen et al., 1998; Hideg et al., 2013). Phenolic compounds are abundant in all plant species and have diverse roles in a variety of environmental responses (Harborne and Williams, 2000). Their synthesis and accumulation in leaves is most frequently observed in plants exposed to UV-B radiation (Stapleton, 1992; Searles et al., 2001). Two major classes of phenolics act as epidermal filters: hydroxycinnamic acids absorbing predominantly in the UV-B and flavonoids with more dominant absorbance in the UV-A (Kolb et al., 2005). In addition, high antioxidant capacities assigned to several phenolic acids and flavonoids *in vitro* (Burda and Oleszek, 2001; Csepregi et al., 2016; Csepregi and Hideg, 2018) suggest that these compounds may also fulfil protective roles *in planta*. Changes in the epidermal UV absorption can be measured directly, in isolated epidermis (Day et al., 1996), inside the leaf using special fibre optics (Bornman and Vogelmann, 1988) or via assessing the inducible chlorophyll fluorescence in intact leaves (Bilger et al., 1997; Goulas et al., 2004). On the other hand, direct evidence for an antioxidant function, such as detecting oxidised flavonoids in leaf tissues, is yet to be found (Hernández et al., 2009). Nevertheless, an array of indirect evidence, such as increased flavonoid levels in mesophyll tissues of sun-exposed *Ligustrum vulgare* leaves (Agati et al., 2009) and the occurrence of flavonoids in chloroplasts (Saunders and McClure, 1976; Agati et al., 2007) and nuclei (Feucht et al., 2004) of several species, support the hypothesis that flavonoids play a key role in countering oxidative stress, and not only in UV screening. Biosynthesis of phenolic compounds in leaves is stimulated by the UV-B via the UVR8 photoreceptor (Rizzini et al., 2011; Morales et al., 2013; Jenkins, 2014), by the UV-A and blue light via the cryptochromes (Wade et al., 2001; Morales et al., 2010; Siipola et al., 2015) and also by low temperature (Suzuki et al., 2005; Bilger et al., 2007; Neugart et al., 2014; Coffey et al., 2017) or desiccation (Suzuki et al., 2005; Bandurska et al., 2013). Low temperature in particular was shown to increase epidermal UV absorption in several, although not all, species studied by Bilger et al. (2007) while most studies refer to changes in leaf total phenolic contents in response to environmental factors.

In a recent study using grapevine (*Vitis vinifera* L.) leaves collected from vineyards along a 1500 km latitude gradient in Europe, we showed that the long-term correlator of leaf flavonoid content was the solar UV radiation received by leaves from bud-break to veraison, rather than the temperature, precipitation, or global photosynthetically active radiation (PAR, 400–700 nm) during the same period (Castagna et al., 2017). The present study is aimed at exploring the environmental drivers on a short-term, hourly scale.

Dynamic responses in epidermal UV absorption to the light environment have been demonstrated in a variety of species (Veit et al., 1996; Sullivan et al., 2007; Barnes et al.,

2008, 2013). Diurnal changes in the epidermal UV transmittance in okra (*Abelmoschus esculentus*) included a transitory mid-day decrease to less than 50% of morning and evening levels (Barnes et al., 2016a). A study of several species in diverse taxonomic families showed that the diurnal adjustments in the UV sunscreen protection are widespread among plants (Barnes et al., 2016b). Grapevine was, however, not included in the above study; and the aim of our work was to investigate whether grapevine leaves displayed a change in the epidermal and the total phenolic contents during the day. We monitored these parameters as well as photosynthesis and antioxidant capacities hourly, for 12 hours, and sought correlations between phenolic profile, physiological parameters and meteorological conditions

2. Materials and Methods

2.1. Plant material and meteorological parameters

The study was carried out at the Szentmiklós Research Station of the Institute for Viticulture and Oenology, University of Pécs, Hungary. Here, twenty-year-old vines of *Vitis vinifera* L. cultivar Pinot Noir are grown on south-facing terraces of the Mecsek Hills (latitude: 46º07' N, longitude: 18º17' E, 200 m elevation) under non-irrigated field conditions. The soil is a Ramann-type brown forest soil mixed with clay, formed on Pannonian red sandstone. Vines are trained to an umbrella system using a 2 m row and a 1 m vine spacing and in east-west-oriented rows. The leaf samples originated from a homogeneous plantation of 150 vines. Mature and healthy, south-facing, sun-exposed leaves from the 7^{th}–10^{th} node were chosen for the analyses. These leaf samples were between 50-80 days old calculated from bud-break phenological stage. Such leaves were shown to be fully developed and acclimated to conditions in the canopy system (Schultz, 1992, 1993; Bertamini and Nedunchezhian, 2002).

The vineyard is situated within the Praeillyricum phytogeographycal district, characterised by 11.6°C annual mean temperature, and receives on an average 782 mm of precipitation and 2021 hours of sunshine annually. However, the 60-year (1950–2010) mesoclimate history of the vineyard includes extreme precipitation (344 and 1140 mm, minimum and maximum, respectively), annual solar hours (1986 and 2548 h) and annual mean temperature (9.3 and 14.0°C) data (Teszlák et al., 2013). Currently, meteorological data are monitored on-site, in the vineyard, using an automatic weather station (WS600, Lufft GmbH, Germany). Air temperature, relative humidity, PAR and broadband UV radiation data are registered every minute. The total UV irradiance data measured on-site were separated into UV-B (280–315 nm) and UV-A (315–400 nm) components for each time point using

UV-A/UV-B ratios, which were calculated with the 5.2 version of the TUV calculator of the National Center for Atmospheric Research – accessible at http://cprm.acom.ucar.edu/Models/TUV/Interactive_TUV/.

2.2. *In situ* photosynthesis measurements

Leaf gas exchange measurements were carried out *in situ* on attached leaves using a portable infrared gas analyser (IRGA) system (LCA-4, ADC BioScientific Ltd., Hoddesdon, UK). Measurements were conducted between 07:00 and 19:00 h local time, corresponding to UTC + 2 h, using ambient (80–2100 μmol $m^{-2}s^{-1}$) PAR and atmospheric CO_2 concentrations. For each leaf, the net CO_2 assimilation, the H_2O transpiration rate, the stomatal conductance and the partial pressure of the intercellular CO_2 (mesophyll conductance) were determined in five technical repetitions. These parameters were calculated from IRGA data by the instrument's software automatically, using the equations of von Caemmerer and Farquhar (1981). Intrinsic water-use efficiency WUE_i was calculated as net CO_2 assimilation divided by stomatal conductance. For correlation analysis, meteorological data that matched the time of each leaf's photosynthesis measurement (as registered with the IRGA instrument) were chosen from the data array of the weather station.

2.3. Non invasive chlorophyll and flavonoid assessment

Following *in situ* photosynthesis measurements, the leaf chlorophyll and the flavonoid indexes were measured at 375 nm using a Dualex® Scientific (FORCE-A, Orsay, France) leaf clip equipment (Goulas et al., 2004; Cerovic et al., 2012). Both indexes were recorded at the adaxial, sun-facing leaf sides. Four measurements were conducted, 2-2 symmetrically on both left and right sides of the mid-vein, avoiding thicker vascular regions. The means of these 4 measurements were used for characterising one leaf. Following the Dualex measurements, the leaves were detached, immediately frozen in liquid nitrogen and stored at -80°C until lyophilisation. Lyophilised leaves were ground to powder using a mixer mill (Retsch MM200, Retsch GmbH, Haan, Germany) and 60 mg leaf powder was extracted into 500 μL pure methanol for 10 min in an ultrasonic cleaner (RoHS JP-020, Shenzhen, China). The extract was centrifuged at 15,000 x g for 10 min at room temperature (Heraeus Fresco 17 Centrifuge, Thermo Fisher Scientific Inc., Waltham, MA, USA), and the supernatant was collected in a reaction tube. This process was repeated twice with 500 μL methanol and the three supernatants per sample were subsequently combined. Supernatants were used in HPLC, total antioxidant and UV absorbing capacity measurements.

2.4. High-performance liquid chromatography analysis

Chromatographic analysis was performed on a PerkinElmer Series 200 HPLC system consisting of a vacuum degassing unit, quaternary pump, autosampler, column thermostat, and a diode array detector (DAD). HPLC separations were achieved by using a Phenomenex Kinetex® 2.6 μm XB-C18 100 Å, 100×4.6 mm column. The column temperature was maintained at 25 °C. The mobile phase comprised (A) 0.1 % formic acid and (B) a mixture of 0.2 % formic acid and acetonitrile (1:1). A flow rate of 1 mL min^{-1} was maintained. The elution program used for the separation is summarised in Supplementary Table S1. Methanolic extract, 5 μL, was injected to the HPLC system and the absorbance was monitored at 330 nm (for caftaric acid) and 350 nm (for flavonols), respectively. High purity reference substances of caftaric acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-glucuronide were obtained from Extrasynthese (Lyon, France). Calibration curves for the quantification were obtained by measuring analytical standards with known concentrations. All compounds were identified by their retention time and UV-Vis spectra. Phenolic, quercetin and kaempferol total contents were calculated as sums of corresponding compounds.

2.5. Total antioxidant and UV absorbing capacity measurements

Antioxidant capacities of leaf extracts were assessed using three different, electron transfer reaction-based methods (reviewed by Huang et al., 2005). Reactivity to the Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927) was determined with a modification of the original method, as described earlier (Csepregi et al., 2013). In this assay, 90 μL of Folin-Ciocalteu reagent (diluted 1:10 in distilled water) was mixed with 20 μL leaf extracts in microplate wells. After a 5-minute incubation at room temperature, 90 μL 6% (w/v) Na_2CO_3 was added to each well. Absorbances at 765 nm were recorded after a 90-minute incubation at room temperature with a plate reader (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). The calibration was made with gallic acid, and Folin-Ciocalteu reactivities of leaf extracts were expressed as μM gallic acid equivalent mg^{-1} dry weight.

The ferric reducing antioxidant potential (FRAP) assay is based on detecting the capacity of leaf extracts to reduce a ferric tripyridyl triazine ($Fe^{(III)}$-$TPTZ_2$) complex to its ferrous form, having an intense bluish colour (Szőllősi and Szőllősi-Varga, 2002). The FRAP

reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in distilled water) and $FeCl_3$ (20 mM $FeCl_3$ dissolved in 40 mM HCl). Extracts of 10 μL, were added to 190 μL FRAP reagent in microplate wells. After a 30-minute incubation at room temperature, the ferrous TPTZ formation was measured at 620 nm using a plate reader. The assay was calibrated with ascorbic acid, and antioxidant capacities of leaf samples were characterised as μM ascorbic acid FRAP equivalent mg^{-1} dry weight.

Trolox equivalent antioxidant capacities (TEAC) of leaf extracts were measured according to Re et al. (1999) with modifications, as described earlier (Majer and Hideg, 2012). This method is based on the reduction of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid cation radical ($ABTS^{\bullet+}$) by antioxidants contained in the sample, resulting in a fading of the blue colour. The TEAC reagent was prepared by incubating ABTS (0.1 mM), horse radish peroxidase (0.0125 mU) and H_2O_2 (1 mM) in a phosphate buffer (50 mM, pH 6.0) for 15 min at room temperature. Extracts of 10 μL were added to 190 μL TEAC reagent in microplate, and absorption was measured at 651 nm. The assay is traditionally calibrated with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and leaf antioxidant capacities are characterised as μM Trolox equivalent mg^{-1} dry weight.

The UV absorbing capacities of the leaf extracts were calculated from absorption spectra, recorded between 280–400 nm, with a spectrophotometer (Shimadzu UV-1800, Shimadzu Corp., Kyoto, Japan). Leaf extracts of 100 μL were added to 900 μL acidified ethanol, and absorption spectra were integrated for the UV-A (315–400 nm) and the UV-B (280–315 nm) regions separately. The integrated absorptions were divided by the widths of the corresponding spectral ranges, 85 and 35 nm, respectively. The total UV-A and UV-B absorptions were given using quercetin aglycone, as standard, in mM^{-1} nm^{-1} as units (Csepregi and Hideg, 2018).

2.5. Statistics

A total of five leaves were collected at each time point, giving a set of 65 data for each parameter. Depending on the parameter, one data corresponded to an individual measurement (HPLC) – an average of three technical replications measured on different aliquots of the same leaf extract (antioxidant capacities or UV absorption) or a mean of biological repetitions measured at different parts of the same leaf (photosynthesis and Dualex measurements). Each n=65 data set was well modelled with a normal distribution, according to the Kolmogorov-Smirnov tests. Correlations between parameters were compared pair wise by calculating the

Pearson's correlation coefficients (R). Significance of each R was determined by testing the null hypothesis of no correlation present in the population against the alternative that there is correlation present. Two data sets were regarded correlated when this test gave $p < 0.05$. Selected parameter pairs showing a strong correlation were also tested using linear regression. A straight line was fitted to these data sets, and the null hypothesis of the slope being equal to zero in the population, was tested against the alternative that the slope is different from zero. Significant ($p < 0.05$) linear correlations were also characterised by the coefficient of determination R^2. Meteorological parameters were strongly correlated (see Results); therefore, multi-linear models using these as independent variables were not applicable to explain changes in photosynthesis or metabolite contents. Calculations were carried out using the PAST software (Hammer et al., 2001). Graphs were prepared using SigmaPlot (Systat Software Inc., San Jose CA USA).

3. Results

3.1. Environmental conditions and physiological parameters

Diurnal changes in environmental parameters are shown in Fig.1. Conditions were ideal for a cloudless summer day at the location (Teszlák et al., 2013; Teszlák et al., 2014). In addition to weather parameters, leaf temperatures were also recorded and showed a mid-day maximum of 40^{o}C (Fig.1D). The south-facing sun-exposed grapevine leaves were well acclimated to the above weather conditions, as evidenced by physiological parameters – shown in Fig.2. Photosynthesis, measured as the net CO_2 uptake, was high in the morning and declined later (Fig.2A) while transpiration followed a bell-shaped pattern and was maximal around solar noon (Fig.2B). Leaf stomatal conductance for water vapour reached a peak level and then decreased gradually from late in the morning throughout the afternoon (Fig.2C), explaining the temporary increase in transpiration at mid-day and the decline of both photosynthesis and transpiration in the evening. Intrinsic water-use efficiency (WUE_i), decreased during the first half of the day, reached a minimum around noon, and increased well above morning levels during the second half of the day (Fig.2D).

3.2. Changes in leaf metabolites during the day

An adaxial UV absorption was assessed at 375 nm (Cerovic et al., 2012) and showed a linear decrease throughout the experiment (Fig. 3A). There was no significant change in the abaxial UV absorption during the same period (data not shown). The adaxial (Fig. 3B) and abaxial (Supplementary Fig.S1A.) leaf chlorophyll index values showed no significant change

during the day. The HPLC analysis showed, in accordance to earlier results (Csepregi et al., 2016; Castagna et al., 2017) that the dominant phenolic acid in Pinot noir leaves was caftaric acid (Fig.4A). It also showed, as new result that caftaric acid contents did not change during the day (Fig.4B). However, the total phenolic and total flavonoid contents increased during the day (Fig.4B), due to a steady increase in the amount of the dominant flavonoid, quercetin-3-*O*-glucuronide (QUE-glu) that counterbalanced a smaller decrease in the quercetin-3-*O*-glucoside (QUE-glc) contents (Fig.4C). The amounts of two other quercetin components, quercetin-3-*O*-rutinoside (QUE-rut) and quercetin-3-*O*-galactoside (QUE-gal), showed no significant change during the day (*p* values for linear time dependences were 0.279 and 0.587, respectively). Kaempferol derivatives gave approximately 6% of the total flavonoid content and were detected as kaempferol-3-*O*-glucuronide (KAE-glu) and kaempferol-3-*O*-glucoside (KAE-glc). While the former was present in the same amount during the day (data not shown), there was a slight but significant increase in the amounts of the latter (p=0.046). In summary, QUE-glc was the only flavonoid component present in smaller amounts in the evening in comparison to the rest of the day. Concentrations of other components either increased during the day (QUE-glu, KAE-glc) or remained the same as in the morning.

3.3. Correlations between environmental conditions, physiological parameters and leaf compositions

Environmental, leaf physiology and metabolite content data were complemented with the total antioxidant and the total UV absorbing capacities of the leaf extracts (Supplementary Fig.S1). Pair wise correlations among the parameters were summarised in a heat map, as shown in Fig.5. Although UV to PAR ratios and UV-B to UV-A ratios vary during the day (Papaioannou et al., 1993), these parameters were strongly and positively correlated. Leaf and air temperatures were positively correlated to each other as well as to irradiance. Relative humidity was negatively correlated to air temperature, as expected, and consequently to irradiance and leaf temperature (Fig.5).

Amounts of major phenolic compounds showed strong positive correlations with each other, with the exception of QUE-glc, which showed positive correlations with minor flavonoids only – but neither with QUE-glu nor caftaric acid (Fig.5). The opposite trend in hourly changes of QUE-glu and QUE-glc (Fig.3C) did not result in a negative correlation between the amounts of these two compounds, presumably due to the differences in their concentrations in leaf extracts. Concentrations of all leaf phenolic components, with the exception of a minor one (KAE-glc), showed strong positive correlations with UV absorbing

capacities of leaf extracts (Fig.5). Equally strong positive correlations were found between antioxidant capacities of leaf extracts, assessed with either the Folin-Ciocalteu or the TEAC assay, and phenolic components.

The adaxial flavonoid index was not correlated to either the total phenolic content or any major phenolic component, indicating that this index reflects a relatively small part of the total content. There was a strong and positive correlation ($p < 0.001$) between the flavonoid index and leaf QUE-glc content (Fig.5), with both the values declining from morning to evening (Fig.3A and 4C). This relationship was significantly linear, as shown in Fig.6A. Among the recorded environmental parameters, irradiation (PAR and UV) conditions and leaf temperature were the only correlators of the flavonoid index. Contrastingly, the same environmental conditions showed no significant correlations with phenolic components, as determined from the total leaf extracts (Fig.5). Although leaf temperature was strongly and positively correlated to air temperature, the two temperature parameters proved to be correlators of distinct leaf features. There was no significant statistical connection between the air temperature and the flavonoid index; however, air temperature was a positive correlator of QUE-glu levels. Because QUE-glu stood for 72 and 75 % of the total quercetin content (lowest and highest for morning and evening compositions, respectively), 67 and 70% of the total flavonoid content and 54 and 56% of the total phenolics, its positive correlation with air temperature also extended to these total content parameters (Fig.5). Figure 6B and 6C show that the distinct environmental correlators of flavonoid index and flavonoid content, irradiation and air temperature, respectively, formed statistically significant linear relationships. The adaxial chlorophyll index showed a strong and negative correlation with the adaxial flavonoid index, and all conditions (PAR, UV, leaf temperature) affecting the flavonoid index positively had a negative effect on the chlorophyll index. QUE-glc, a strong and positive correlator of the flavonoid index, showed a strong and negative correlation with the chlorophyll index. However, the correlators of the two indexes were not completely opposites: air temperature that showed no statistical connection to the flavonoid index was a negative correlator of the chlorophyll index (Fig.5). Additionally, several other minor flavonoid components were negatively correlated with the chlorophyll index. Interestingly, although neither the dominant flavonoid nor the caftaric acid showed up as correlators, the total phenolic content was negatively correlated to the chlorophyll index (Fig.5).

4. Discussion

Statistical analyses of our data set suggest that short-term daily changes in the adaxial flavonoid index are regulated by different environmental factors than those controlling the amount of flavonoids measured in the total leaf extracts. Due to the technical difficulties of removing the epidermal layer from the grapevine leaves, a separate HPLC profiling of these tissues was not possible. Likewise, the penetration depth of the 375 nm UV-A applied for assessing the flavonoid index is unknown; therefore we will be relying on our assumptions. Three arguments, (i) the ca. 2% relative thickness of the upper epidermis compared to the whole leaf's cross section in Pinot noir sun leaves (Boso et al., 2010), (ii) the lack of correlation between the flavonoid index and the amount of any identified phenolic components but QUE-glu as well as (iii) the lack of correlation between UV-A absorption of the whole leaf extract and the flavonoid index in the present study, suggest that the quantitative ratio of phenolic compounds responsible for the epidermal UV-A absorbance to those located in the rest of the leaf is very small. Nevertheless, it is well established that the epidermal UV absorbing compounds are of key importance for the whole leaf (Bilger et al., 2007). When assessed with a different instrument (UV PAM, Kolb et al., 2005), utilising the same physical principle as the one applied in our study (Goulas et al., 2004), Barnes et al. (2016b) investigated diurnal changes in epidermal UV transmittance in a number of samples and identified unresponsive and responsive species and cultivars. In their study, responsive leaves showed a diurnal decrease in epidermal UV transmittance (Barnes et al., 2008; 2016b), which would correspond to an increase in UV absorption if measured with the instrument used in the present study. Grapevine was not among the species studied by Barnes et al. (2016b), and our results showed a small but statistically significant 7% decrease in the flavonoid index from the morning mean 1.94 to the evening mean 1.81 (Fig.3A), identifying the opposite of the reported trend. It should be noted that a comparison of the data at these two time points (morning and evening only) would not have identified a significant difference due to the large variation among individual leaves sampled at the same time of the day. A regression analysis of 65 data pairs (time, flavonoid index) was necessary to establish the observed trend (Fig. 3A). In this respect, the Pinot noir grapevine leaves are either unique and do not fall into the two categories established by Barnes et al. (2016b) or more likely categorised as unresponsive by the method applied in their study. Further analysis of diurnal changes in grapevine leaf flavonoid index, including pre-dawn measurements of this parameter will be necessary to classify the dynamics of epidermal UV transmittance of this species.

We found that variation in grapevine adaxial flavonoid index was positively correlated with solar UV-B and PAR, in accordance with studies using okra leaves (Barnes et al., 2015; 2016a). As a novel result, our data also identified solar UV-A and leaf temperature as positive correlators. Air temperature, implicated as a factor affecting epidermal UV transmission in several species (Barnes et al., 2016b), had no significant effect on the flavonoid index in our study. On the other hand, air temperature was a positive correlator of total extractable phenolic content, through increasing levels of the dominant flavonoid, QUE-glu (Fig.6D). It is unclear why the epidermal flavonoid index and whole leaf phenolic constituents are distinct in environmental correlators when the biosynthesis of these compounds is similar. A possible explanation is based on the strong and positive correlation found between the flavonoid index and QUE-glc content (Fig. 6A). Assuming that the ratio of epidermal and sub-epidermal QUE-glc is higher than that of other flavonoids, this flavonol would support keeping guard cell ROS levels low by serving as a phenolic peroxidase substrate and thus enabling functional stomata responses. This hypothesis is supported by reports on (i) quercetin-3-triglucoside in the upper epidermal guard cells of *Pisum sativum* (Weissenböck et al., 1986), (ii) functional phenolic peroxidase in grapevine leaves (Perez et al., 2002) and (iii) indications of vacuolar peroxidase activity in grapevine (Zipor and Oren-Shamir, 2013). The observed decreased in QUE-glc (Fig.3C) may indicate the oxidation of this flavonol in the epidermis either directly by ROS or as a result of electron donation to phenolic peroxidases. Because oxidised flavonol-glycosides can be recovered by ascorbate (Yamasaki et al., 1997), an even higher rate of QUE-glc oxidation is also feasible. A preferential localisation for at least part of QUE-glc in the epidermis is yet to be proven experimentally, as well as the identification of oxidised flavonols *in planta*. The above hypothesis would explain different environmental correlators of QUE-glu and QUE-glc (Fig.5). Positive correlations of both photosynthesis and internal sub-stomatal CO_2 concentration along with the flavonoid index and the QUE-glc content (Fig.5) support the idea of this flavonol having a unique role.

Moreover, positive drivers of the flavonoid index (PAR, UV and leaf temperature) showed no connection with phenolic compound levels other than that of QUE-glc. However, contributions of other, non-extractable epidermal polyphenols to short term changes in the flavonoid index, such as p-coumaric and ferulic acids found in NaOH-hydrolysed leaf pellets of the Garciano grapevine cultivar (Del-Castillo-Alonso et al., 2015) cannot be excluded. A negative correlation between the chlorophyll and phenolic contents (Fig.5) indicates possible metabolic costs of short-term, dynamic changes similarly to the trend found in long-term, constitutive responses (Castagna et al., 2017).

In our experiment, the photosynthetic CO_2 uptake was positively correlated to PAR and negatively to temperature parameters (Fig.5). Such negative temperature dependence has already been demonstrated in the leaves of other red grape varieties (Escalona et al., 2012; Carvalho et al., 2016). The observed positive correlation between photosynthesis and internal CO_2 concentration is a common characteristic of the grapevine leaves (Flexas et al., 2009). Transpiration and leaf temperature were positively correlated (Fig.5), in accordance to the data of other cultivars (Keller, 2010). Increased air water vapour deficit (registered as decreased RH in our experiment) has been identified as a strong driving factor of stomatal closure in the grapevine leaves (Escalona et al., 2012; Flexas et al., 2002; 2009), explaining the positive correlations found between RH and stomatal closure, RH and internal CO_2 concentration as well as RH and photosynthesis (Fig.5). WUE_i was relatively low, indicating water stress, especially late morning (Medrano et al., 2015). On the other hand, transpiration and stomal conductance were also positively correlated, indicating that this water-stress was not severe (Chaves et al., 2016; Marchin et al., 2016). Also, the observed daily minimum ratio of photosynthesis and transpiration, approx. 1.5 μmol CO_2/mmol H_2O between 11h and 13h (calculated from data in Fig.2), was characteristic to a physiological status between the well-watered and the water-stressed grapevines (Poni et al., 2014). Leaves in our experiment undoubtedly responded to hourly changes in their water status by adjusting the synthesis of various metabolites. However, neither epidermal UV-A absorbing compounds nor extractable phenolic components were among these metabolites, as indicated by the lack of correlations between WUE_i and these parameters (Fig.5).

Photosynthetic CO_2 uptake was negatively correlated to solar UV, both UV-A and UV-B. Because PAR was a positive correlator in our experiment, the negative effect of UV cannot be explained as an effect carried over from the strong positive PAR–UV correlation (Fig.5). This putative negative effect of UV was not realized via limitations of either the stomatal conductance or the internal sub-stomatal CO_2 concentration, as these two parameters are positively correlated to all radiation parameters, including UV (Fig.5). It is generally agreed that the direct, damaging effects of ambient solar UV on photosynthesis are minor in crop plants (Fiscus and Booker, 1995, Ballaré et al., 2011). These reports, however concern long-term responses. The observed negative UV effect on photosynthesis reported here is distinct from the strong inhibition of CO_2 assimilation brought about by a sudden sun exposure of the shade-acclimated leaves observed in tropical tree seedlings (Krause et al., 2003), and we suggest that it is rather a short-term down-regulation, than an inhibition.

Strong positive correlations were found between leaf total antioxidant capacities (measured as TEAC or FCR) and phenolic contents. These results suggest an *in situ* antioxidant function for at least some of these compounds, although the applied methods do not allow assignment of the antioxidant function to tissue-specific localisation. Results of the FRAP assay were only correlated with the caftaric acid content, that is in line with different performances of various phenolic components in different assays. In an earlier study, we found that caftaric acid was about 3-times more reactive to the FRAP than to the TEAC reagent while corresponding reactivities of quercetin-glycosides are only twice. Moreover, KAE-glc and KAE-glu were found to be nearly unreactive in the FRAP assay while their TEAC values were 80–85% of their corresponding quercetins (Csepregi et al., 2016).

Our findings further indicate a positive correlation between the leaf total antioxidant capacity and the solar UV (both UV-A and UV-B). These correlations and the above discussed negative effect of UV on photosynthesis offers a hypothesis that links the two findings. Grapevine leaves with limited stomatal conductance increase photorespiration (Flexas et al., 2002, reporting a 0.15 mmol CO_2 passage m^{-2} s^{-1} limit), and H_2O_2 from photorespiration might serve as messenger molecule to trigger higher antioxidant defence. In this way, the internal CO_2 concentration becomes a negative correlator of the leaf antioxidant capacities, as observed in our experiment (Fig.5). UV-B radiation from artificial sources was shown to increase the H_2O_2 contents of *Arabidopsis* (Czégény et al., 2014) or *Oryza sativa* (Dai et al., 1997) leaves in model experiments. In these experiments, the H_2O_2 source was not the peroxisome, as indicated by peroxidase rather than catalase responses to supplementary UV-B (Czégény et al., 2016). Assuming a similar role of solar UV in grapevine leaves would explain the observed positive correlation with the total antioxidant capacity. Although rates of the Mehler reaction in grapevine leaves were found to be low in the absence of chilling or desiccation stress (Flexas et al., 1999), small amounts of chloroplast-derived H_2O_2 would play a similar signalling role as the peroxisomal ROS. Using *Arabidopsis* mutants, Sewelam et al. (2014) showed the existence of responses that are independent from the subcellular site of H_2O_2 production, in addition to specific signals depending on the origin of ROS. Whether such integrated signals exist in sun acclimated grapevine leaves is the subject of future studies, as well as the identification of non-enzymatic antioxidant compounds that are responsive to the above hypothesised metabolic signalling. The correlation map of our data set implies that these compounds have relatively lower UV absorption and higher reactivity to the TEAC than other total capacity assays. Ascorbate, with its equal TEAC and FRAP reactivity (Csepregi et al., 2016), is an unlikely candidate. Glutathione, which is unreactive to

the FRAP assay (Prior et al., 2005) but has a high TEAC (Re et al., 1999), and carotenoids having much lower FRAP than TEAC reactivity (Müller et al., 2011) are among possible compounds.

5. Conclusions

High base levels of phenolic components, present in grapevine leaves as a result of long-term adaptation, are not constant during the day but are modulated by complex radiation and temperature signals originating in environmental factors. Contrary to the extensive changes in phenolic profiles observed in a variety of plants under modulated sunlight or low temperature, only a relatively small fraction of grapevine leaf phenolic compounds was responsive to dynamic changes in the natural environment in our experiment. Epidermal UV-A absorbance, characterized by the flavonoid index, responded to different factors than whole leaf flavonoids, indicating diverse roles according to micro-localization. The inverse relationship between CO_2 uptake and the total antioxidant capacity, together with the positive correlation between CO_2 uptake and the flavonoid index suggest the possibility of dynamic re-allocation of quanta among photosynthesis, defence and protection.

Acknowledgements

Experimental work was supported by the National Research, Development and Innovation Office (grant number NKFIH K124165) and by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. The project was also supported by the European Union, co-financed by the European Social Fund Grant no.: EFOP-3.6.1.-16-2016-00004 entitled by Comprehensive Development for Implementing Smart Specialization Strategies at the University of Pécs. K.Cs. wishes to thank the Hungarian Society of Plant Biologists for the Scientia Amabilis Foundation Scholarship.

Figure Captions

Figure 1

Changes in environmental parameters and leaf temperature during the experiment.

Figure 2

Changes in Pinot noir leaf photosynthesis and water use parameters during the experiment.

Figure 3

Changes in (A) adaxial epidermal UV-A absorption and (B) chlorophyll content during the experiment.

Both parameters were measured non-invasively as Dualex flavonoid and chlorophyll index, respectively. Straight lines show results of linear fits for n=65 data; numbers in parenthesis are the coefficient of determination R^2, and the *p* value characterizing the statistical significance of a linear relationship.

Figure 4

Changes in Pinot noir leaf phenoloid profiles during the experiment.

Three time points show phenoloid compositions in the morning, close to solar zenith and in the evening (A). Pie chart areas are proportional to the total phenolic contents. QUE-glu, quercetin-3-*O*-glucuronide; QUE-glc, quercetin-3-*O*-glucoside; QUE-rut, quercetin-3-*O*-rutinoside; QUE-gal, quercetin-3-*O*-galactoside; KAE-glu, kaempferol-3-*O*-glucuronide; KAE-glc, kaempferol-3-*O*-glucoside.

Changes in the total phenolic, total flavonoid, caftaric acid (B), QUE-glu, and QUE-glc (C) contents during the day were characterised by linear models, as results of regression analyses, using the least squares method. Numbers in parenthesis are the coefficient of determination R^2, and the *p* value characterizing the statistical significance of a linear relationship.

Figure 5

Correlations between environmental conditions, leaf physiology, metabolite content and antioxidant capacity parameters.

Pearson's correlation coefficients were calculated pair wise for two n=65 data sets. Colours corresponding to positive (red, orange, yellow) or negative (dark blue, cyan, pale blue)

correlations at *p* levels are shown in the figure. White cells indicate the lack of significant correlation.

Environmental conditions: PAR, photosynthetically active radiation; sol UVA+B, solar UV radiation 280–400 nm; sol UVA, solar UV-A radiation 315–400 nm; sol UVB, solar UV-B radiation 280–315 nm; air T, air temperature; RH, relative air humidity.

Leaf physiology parameters: leaf T, leaf temperature; phot CO_2, photosynthetic CO_2 uptake; transp H_2O, water vapour transpiration; WUE_i, intrinsic water-use efficiency calculated as photosynthetic CO_2 uptake divided by stomatal conductance; g-stomata, stomatal conductance to CO_2; intern CO_2, internal sub-stomatal CO_2 concentration.

Metabolite contents determined with HPLC: tot Phen, total phenolic content; CA, caftaric acid; tot Flav, total flavonoid contents; QUE-glu, quercetin-3-*O*-glucuronide; QUE-glc, quercetin-3-*O*-glucoside; QUE-rut, quercetin-3-*O*-rutinoside; QUE-gal, quercetin-3-*O*-galactoside; KAE-glu, kaempferol-3-*O*-glucuronide; KAE-glc, kaempferol -3-*O*-glucoside.

Antioxidant capacities of leaf extracts: aox FC, total antioxidant capacity measured with the Folin-Ciocalteu method; aox TEAC, total antioxidant capacity measured with the TEAC method; aox FRAP, total antioxidant capacity measured with the FRAP method (see Materials and Methods for details).

Other, metabolite-related parameters: abs UVA+B, total 280–400 nm absorption of leaf extracts; abs UVA, 315–400 nm absorption of leaf extracts; abs UVB, 280–315 nm absorption of leaf extracts; indx Flav, adaxial leaf flavonoid index; indx Chl, adaxial leaf chlorophyll index.

Figure 6

Linear dependences between flavonoid index, phenolic contents and environmental parameters.

Environmental conditions: PAR, photosynthetically active radiation; Solar UV, 280–400 nm radiation. Metabolite contents determined with HPLC: Tot Phen, total phenolic content; QUE-glu, quercetin-3-*O*-glucuronide; QUE-glc, quercetin-3-*O*-glucoside. Lines represent linear models, as results of regression analyses using the least-squares method. Numbers in parenthesis are the coefficient of determination R^2, and the *p* value characterizing the statistical significance of a linear relationship.

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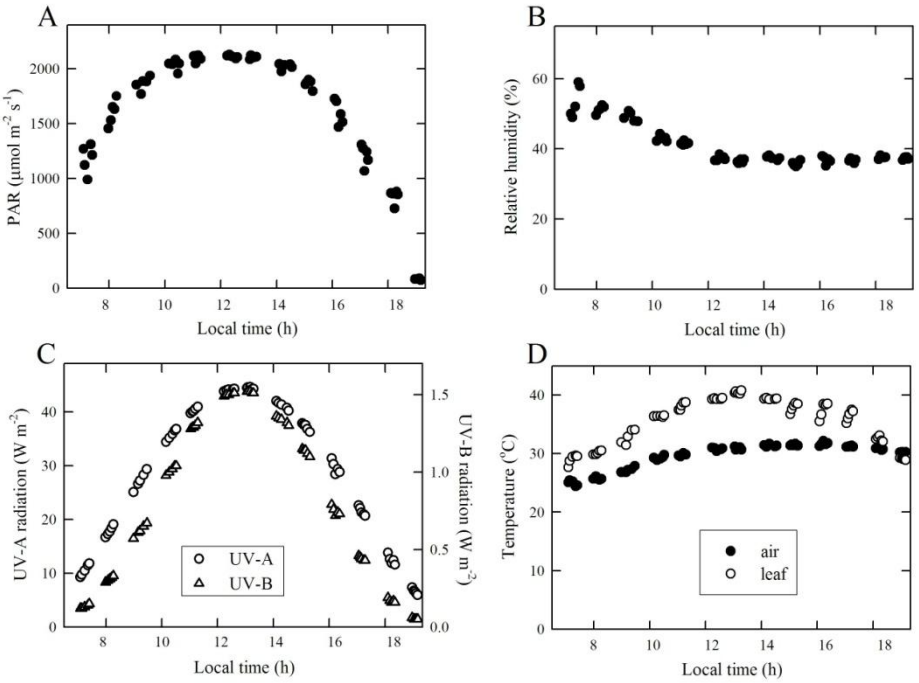

Figure 1

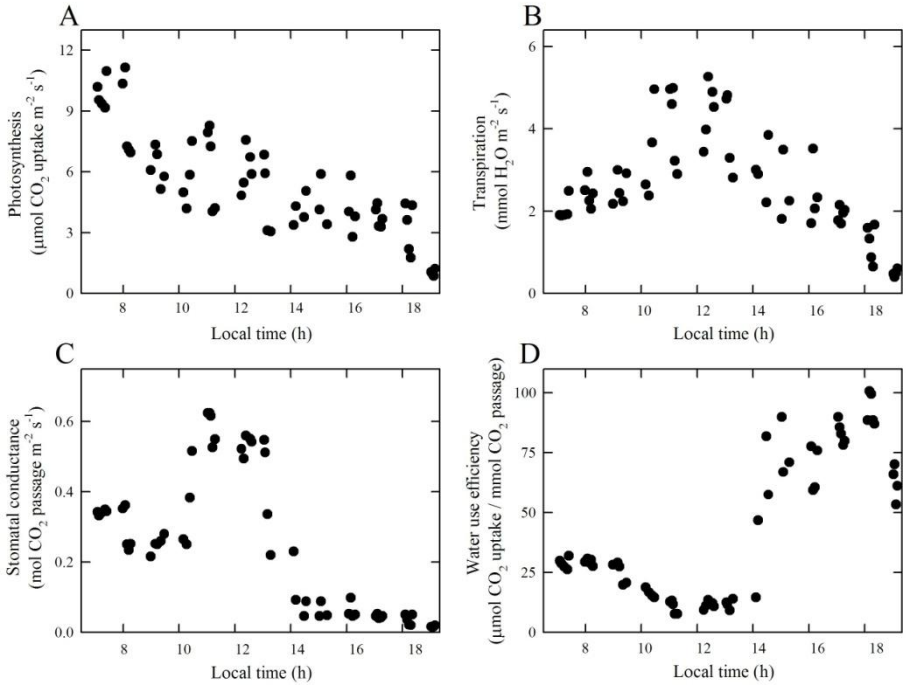

Figure 2

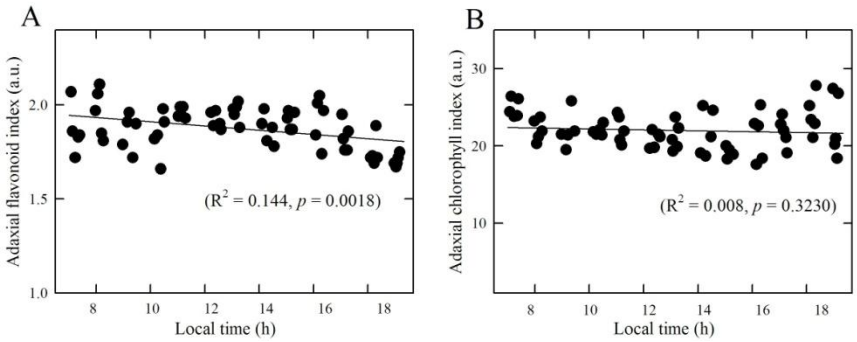

Figure 3

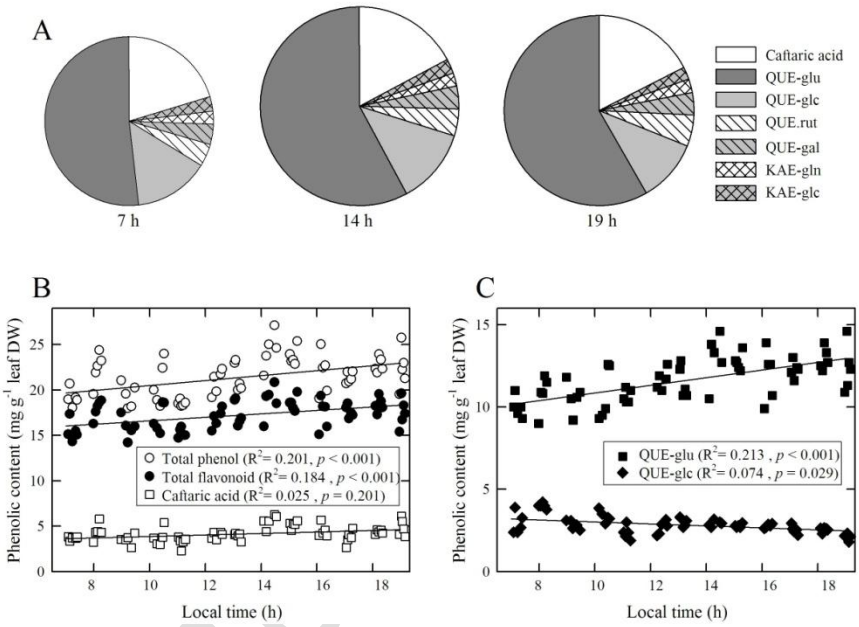

Figure 4

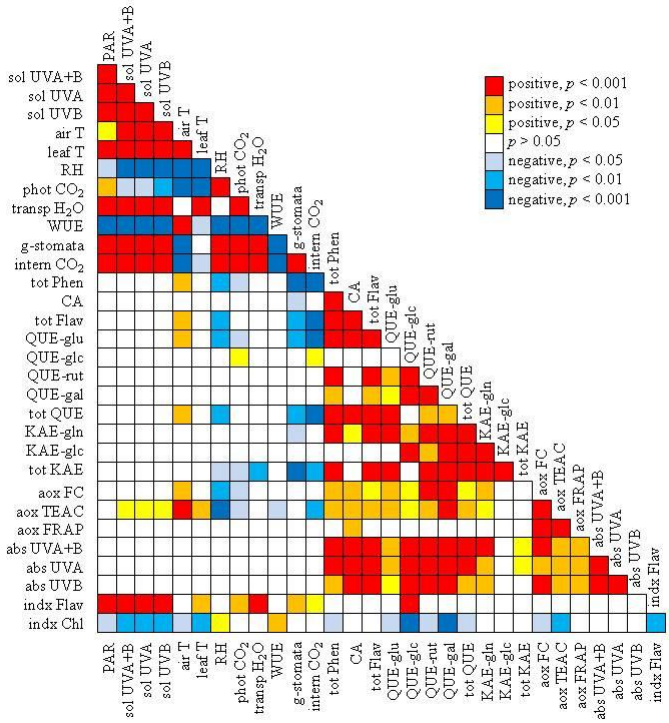

Figure 5

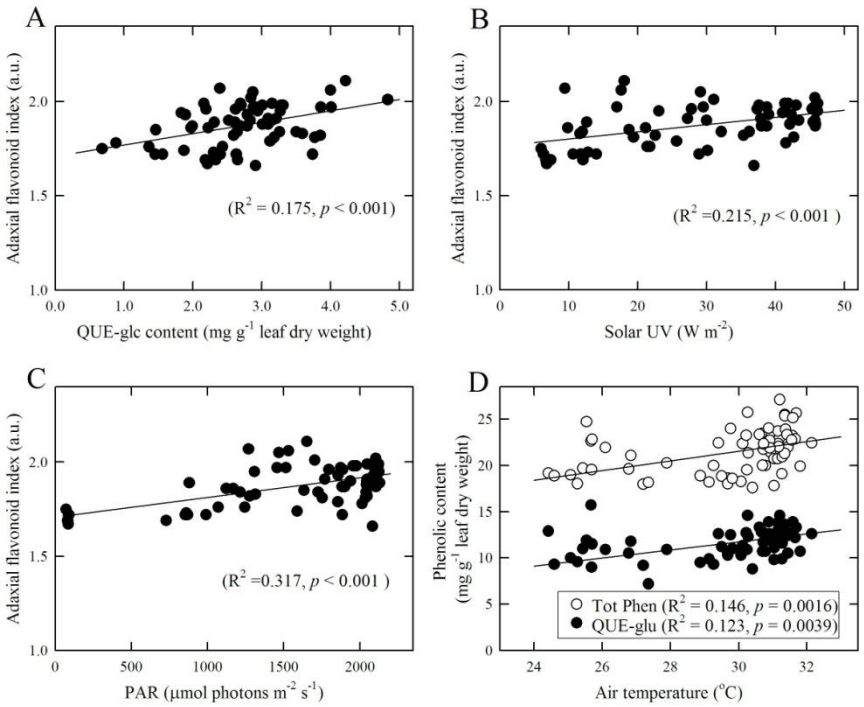

Figure 6