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Constitutive changes in pigment concentrations: Implications for estimating isoprene emissions using the photochemical reflectance index (PRI)

A. Harris[1], S. M. Owen[2], D. Sleep[3], M. G. Pereira[3]

[1]Geography, School of Education, Environment and Development, The University of Manchester, Manchester, M13 9PL, UK

[2] Centre for Ecology & Hydrology, Bush Estate, Penicuik, EH26 0QB, UK

[3] Centre for Ecology & Hydrology, Lancaster Environment Centre, Lancaster, LA1 4YQ, UK

Author for correspondence: *Angela Harris*
angela.harris@manchester.ac.uk

Abstract

The photochemical reflectance index (PRI), through its relationship with light use efficiency (LUE) and xanthophyll cycle activity, has recently been shown to hold potential for tracking isoprene emissions from vegetation. However, both PRI and isoprene emissions can also be influenced by changes in carotenoid pigment concentrations. Xanthophyll cycle activity and changes in carotenoid concentrations operate over different timescales but the importance of constitutive changes in pigment concentrations for accurately estimating isoprene emissions using PRI is unknown. To clarify the physiological mechanisms behind the PRI-isoprene relationship, the light environment of potted *Salix viminalis* (dwarf willow) trees was modified to induce acclimation in photosynthetic rates, phytopigments, isoprene emissions and PRI. Acclimation resulted in differences in pigment concentrations, isoprene emissions and PRI. Constitutive changes in carotenoid concentration were significantly correlated with both isoprene emissions and PRI, suggesting that the relationship between PRI and isoprene emissions is significantly influenced by constitutive pigment changes. Consequently knowledge regarding how isoprene emissions are affected by both longer term changes in total carotenoid concentrations and shorter term dynamic adjustments of LUE is required to facilitate interpretation of PRI for monitoring isoprene emissions.

Abbreviations

A, antheraxanthin; βC, β-carotene; BVOC biogenic volatile organic compounds; Car, carotenoids; EPS, epoxidation state; L, lutein; LUE, light use efficiency; N, neoxanthin; PRI, photochemical reflectance index; V, violaxanthin; Z, zeaxanthin.

Introduction

Biogenic volatile organic compounds (BVOCs) are a chemically reactive carbon flux and thus play an important role in global atmospheric chemistry. BVOCs affect the distribution and residence time of short-lived radiatively active trace gases such as tropospheric ozone (O_3) and methane (CH_4; Fiore et al., 2012). Land-based vegetation returns about 1 PgC of total BVOC emissions to the atmosphere each year (Guenther et al., 2012). The reasons why plants invest in BVOC emissions remains unclear, although ecological and physiological roles of emissions are thought to include the ability to attract pollinators and decrease pathogen attacks and herbivory (Gershenzon, 1994; Michelozzi, 1999; Niinemets et al., 2013); to increase leaf thermotolerance (Singsaas et al., 1997); and to protect the plant against oxidative stress (Vickers et al., 2009).

Isoprene is the most dominant BVOC emitted by plants, representing almost half of the total annual flux of reactive carbon (Guenther et al., 2012). Whilst it is generally accepted that anthropogenic and natural perturbations to isoprene emissions are likely to have an important influence on regional climates and feedbacks to global climate (Pitman et al., 2012), there is a lack of quantitative understanding of the mechanisms controlling patterns of emissions over long timescales (weeks to months; Porcar-Castell et al., 2009) and across regions (Foster et al., 2014), which makes modelling emissions challenging. Furthermore, many isoprene emission models, both empirical and process-based, base estimations on linkages between isoprene emissions and plant primary productivity (Arneth et al., 2007; Foster et al., 2014; Guenther et al., 1993), even though it is known that isoprene and photosynthetic activity can become decoupled under conditions such as high temperatures (Unger et al., 2013); during drought stress (Monson et al., 2007; Niinemets et al., 2010); under increasing atmospheric CO_2 concentration (Monson et al., 2007; Rosenstiel et al., 2003); and due to the presence of time lags between the seasonal onset of photosynthesis and isoprene emissions (Monson et al., 1994; Pressley et al., 2005). As a consequence, there is a need to base isoprene estimations on fundamental links between emissions and the biological processes that affect these emissions. Emission models such as the Model of Emissions of Gases and Aerosols from Nature (MEGAN; Guenther et al., 2006), go some way towards achieving this aim, but they are increasingly complex and uncertainty in their estimations can be high (Guenther et al., 2006).

Recently Peñeulas *et al.* (2013) suggested a simple approach for estimating isoprenoid (i.e. isoprene and monoterpene) emissions using remotely sensed data. Unlike many previous attempts at using

remote sensing to estimate isoprenoid emissions, which focus on the detection of formaldehyde (an isoprenoid oxidation product) in the atmosphere (Barkley et al., 2008; Foster et al., 2014; Palmer et al., 2003), Peñuelas et al. (2013) showed that a simple spectral index (the photochemical reflectance index; Gamon et al. 1992) that is indicative of changes in plant light use efficiency (LUE), when combined with basal emission factors, was as good a predictor for isoprenoid emissions as some standard emission models (Peñuelas et al., 2013). Furthermore, the high temporal resolution and spatially extensive nature of remotely sensed data can help capture some spatial and temporal variability in emissions that other models may miss (Peñuelas et al., 2013).

The use of LUE as an indicator of isoprene emissions is based on the idea that when LUE is low (e.g. under conditions of high irradiance), photosynthesis is reduced and thus more reducing power (NADPH) is available for isoprene production (Morfopoulos et al., 2013). Previous studies have also shown strong links between LUE and the photochemical reflectance index (PRI) due to changes in the levels of photoprotective xanthophyll cycle pigments in response to excess irradiance, which can be detected through changes in leaf reflectance (Gamon et al., 1992; Peñuelas et al., 1995). In the short term (seconds to hours), conversions of xanthophyll cycle pigments between their epoxidised and de-epoxidised states (e.g. conversion of violaxanthin to zeaxanthin via antheraxanthin; Demmig-Adams and Adams, 1992) results in rapid, and typically temporary, facultative PRI changes that scale with LUE (Gamon et al., 1992; Peñuelas et al., 1995). Whilst at these time scales, PRI and LUE are closely correlated at the leaf-scale, at longer time scales (weeks to months) correlations between LUE and PRI are often variable as other factors besides xanthophyll pigment conversion may be driving the PRI signal (Filella et al., 2009; Gamon et al., 1997; Porcar-Castell et al., 2012; Wong and Gamon, 2014). Similarly, at longer time scales, isoprene emissions are also thought to be influenced by environmental factors other than temperature and light (Geron et al., 2000; Harley et al., 1996; Pressley et al., 2005). In a series of unrelated studies, at longer time scales changes in the carotenoid concentration has been shown to influence both PRI, through reflectance changes caused by changes in the chlorophyll to carotenoid pigment ratio (Gamon and Berry, 2012; Wong and Gamon, 2014), and isoprene emissions, thought to be caused by either substrate availability or complementary functionality (Owen and Peñuelas, 2013).

The correlations between PRI and isoprene emissions observed by Peñuelas *et al*. (2013), which are based on LUE (through relationships between xanthophyll pigment conversions and LUE), are thus likely to be influenced by facultative changes in xanthophyll cycle pigments. The extent to which constitutive pigment concentrations also influence the PRI-isoprene emission relationship has not been explicitly studied, but may be important for seasonal monitoring of emissions, and under conditions where changes in LUE and pigment concentrations vary asynchronously. The main aim of this study is thus to investigate the effect of constitutive changes in pigment pools on the PRI –

isoprene emission relationship. In doing so we assess some of the physiological mechanisms behind the relationship, primarily through understanding the influences of constitutive differences in pigment concentrations, and facultative differences in xanthophyll pigment conversions, on both isoprene emissions and the PRI reflectance signal.

Materials and methods

The relationships between PRI, isoprene emissions, photosynthetic rates and phytopigments were investigated in the tree species *Salix viminalis* (Dwarf Willow). Willows were chosen as they are known to be strong emitters of isoprene (Kesselmeier and Staudt, 1999) and are also widely recognised as suitable bioenergy crops (Karp and Shield, 2008; Keoleian and Volk, 2005; Larsson, 1998). Consequently, Willows play a potentially important role in the future global rate of isoprene emissions (Lathiere et al., 2010).

Plant material and sampling strategy

The experiment was performed at the Centre for Ecology & Hydrology (CEH) Edinburgh, UK, during the end of July 2014 when natural daylight extends from ~ 05:15 to 21:15. Potted *S. viminalis* plants (approximately 1-2 years old) were obtained from a commercial nursery (http://www.treesbypost.co.uk) at the beginning of June 2014. The saplings were potted into compost in 6.5 litre pots. To widen the range of isoprene emissions and pigment concentrations tested, each of the twenty four plants were subsequently transferred to one of three natural light environments in the grounds of CEH, Edinburgh. Eight plants were kept against a south-facing wall, which received full sun (SUN), eight were kept against a south-east facing wall, which was shaded for half of the day (HALFSHADE) and eight were kept in a naturally full shaded location, with the addition of a double layer tent of horticultural netting to produce deep shade (SHADE). During the eight week period, generally sunny conditions prevailed and 30% of the continuous 30-minute PAR measurements were greater than 500 µmol m^{-2} s^{-1}. All plants were kept well-watered until the measurements commenced approximately 8 weeks later.

Sampling was undertaken over the course of a three day period i.e. one sampling day for plants exposed to each of the three different light environments. Leaves of equivalent size and maturity were selected for each plant. Prior to sampling, each plant in a given treatment was transferred to a dark room and covered with a black shade cloth for ~ 40 minutes to ensure that leaves were in a dark-adapted state prior to reflectance sampling. PRI from dark-adapted leaves has previously been shown to relate to constitutive changes in pigment concentrations and thus by using PRI values obtained

from dark-adapted leaves we minimise confusion of the PRI interpretation caused by facultative changes in xanthophyll pigment pools, which occur as a consequence of diurnal acclimatisation (Gamon and Berry 2012; Porcar-Castell et al., 2012).

Measurements for each plant leaf were undertaken in the following order: 1) spectral reflectance, 2) isoprene emission measurements and 3) pigment analyses. All sampling was undertaken between the hours of 09:00 – 17:30.

Spectral reflectance measurements

Leaf reflectance was measured using a spectroradiometer (FieldSpec Jr; ASD, Boulder, CO, USA) equipped with a fibre optic, a leaf clip and contact probe (ASD), which enabled all reflectance measurements to be collected under fixed geometric and illumination conditions. The contact probe probe's light source is a halogen–krypton bulb with peak irradiance at a wavelength of ~ 966 nm. The spot size was 10 mm, and the sampling interval and spectral resolution of the instrument was 1.4 nm and 3 nm; respectively. The integration time was 68 ms and to avoid any light acclimatisation during measurements, spectra were averaged over 5 measurements only. To calculate reflectance, each leaf spectra was divided by a white reference measurement obtained from a calibrated Spectralon® (Spectralon, LabSphere, North Sutton, NH, USA) reference panel immediately prior to each set of leaf measurements. The Photochemical Reflectance Index (PRI) was calculated as follows:

$$PRI = (R_{531} - R_{570})/(R_{531} + R_{570})$$

Where R is reflectance and the subscript indicates the wavelength (nm; Gamon et al., 1992; Peñuelas et al., 1995).

Isoprene emission sampling and analysis

Isoprene emission measurements were made using an ADC LCpro leaf cuvette. The flow rate through the cuvette was 200 umol s^{-1} (~300 ml min^{-1}). The temperature inside the cuvette was set to 25 °C and the PAR was 1000 umol m^{-2} s^{-1}. On each occasion, the sampled leaf was installed in the cuvette and equilibrated for 20-30 minutes to allow dynamic equilibrium of photosynthesis and isoprene emission rates within the cuvette (Geron et al., 2006). A sample of the air exiting the cuvette was drawn through a stainless steel tube containing Tenax and Carbotrap to trap emitted volatile organic compounds (VOCs), using an SKC mass flow controlled pocket pump at 200 ml min^{-1}.

At the end of the sampling, the leaf area inside the cuvette was marked and leaf samples of 4 mm diameter were extracted and frozen in liquid nitrogen for pigment analysis using high performance liquid chromatography (HPLC; see section *Pigment analyses*). The outline of the leaf was subsequently traced onto paper, cut out, and the leaf area determined by weighing the paper template after calibration of the paper. Due to the simple structure of the leaves, there was < 5 % uncertainty associated with this method of determining leaf area.

Isoprene emission samples were desorbed using a Perkin Elmer automatic thermodesorption device (Turbomatrix™ ATD), into a gas chromatograph-mass spectrometer (Perkin Elmer GC-MS) with Helium as carrier gas. Compounds were desorbed at 280 ˚C for 6 minutes onto a Tenax-TA cold trap, which was maintained at -30 ˚C. The trap was then flash-heated to 280 ˚C for 5 minutes secondary desorption onto the GC column. The GC column was held at 35 ˚C for 2 minutes, then heated to 160 ˚C at 4 ˚C min^{-1}, followed by a final heating to 300 ˚C at 45 ˚C min^{-1}. The temperature was held at 300 ˚C for 10 minutes. Isoprene quantification was achieved by injecting and analysing 30 ml of 0.7 ppm gaseous isoprene standard (Air Products) onto adsorbent tubes and analysed in the same way as the samples. Quality assurance standards were analysed at the start of the batch, and then for every 5 samples.

Pigment analyses

Phytopigments in the frozen (-80 °C) leaf discs from the sampled willow leaves were extracted into acetone (buffered with $CaCO_3$), after grinding under liquid nitrogen in a mortar and pestle. Extracts were centrifuged (5000 rpm for 5 minutes) and the supernatant filtered through a 0.2 micron PTFE syringe filter. Prior to HPLC analysis, extracts were diluted 3:7 with the aqueous component (70 % MeOH plus 30% 28 mM Tetra butyl ammonium acetate TBAA) of the HPLC mobile phase to achieve a good chromatographic peak shape. A variable aliquot (20-200 µl) was injected into the HPLC (Agilent 1100), using a reverse phase chromatographic Agilent Zorbax Eclipse XDB 8 (3.0 x 150 mm, 3.5 µm particle size @ 60 °C).

Absorbance was measured at: 440 nm, 450 nm, 470 nm, 480 nm and 665 nm depending on the pigment. The complete spectrum of photosynthetic pigments in the 370-750 nm ranged was carried out to confirm identification

External standards (DHI Lab products, Hørsholm, Denmark), duplicates and blanks were used for Identification and quantification. The linear gradient of solvent elution for quantification is shown in Table 1.

Xanthophyll cycle pigment pools (VAZ) were calculated as the sum of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) concentrations. Total carotenoid concentration (Car) was calculated as the sum of neoxanthin (N), V, A, Z, lutein (L) and β-carotene (βC). Total chlorophyll (Chl) was calculated as the sum of Chlorophyll *a* and *b* concentrations. Pigments were expressed individually on an area basis (μmol m^{-2}) and carotenoids were also normalised to total chlorophyll concentration (mmol mol^{-1}). The epoxidation state of the xanthophyll cycle, which is an expression of the non-photoprotective pigment composition of the xanthophyll cycle, was calculated as:

$$EPS = \frac{V + 0.5A}{V + A + Z}$$

Statistical analyses

Differences between the three light environment groups were analysed using one-way ANOVA with Tukey's honestly significant difference (HSD) post-hoc test. We calculated the Pearson's correlation coefficient (*r*) to evaluate relationships between variables. All statistical analyses were undertaken in the R statistical software package (R Development Core Team 2012).

Results

Isoprene emission potentials, phytopigments and PRI of leaves acclimatised to sun, shade and half-shade

Leaves acclimatised to different illumination conditions showed significant differences in their pigment pools, isoprene emissions, photosynthetic rates and recorded PRI values (Table 2).

SUN leaves exhibited higher concentrations of total carotenoids ($F_{(2,21)}$ = 33.11, $P < 0.001$), and xanthophyll cycle pigments ($F_{(2,21)}$ = 118, $P < 0.001$) per area than SHADE leaves. Chlorophyll concentration per area was highly variable within leaves of a given treatment (data not shown) and thus observed differences were only significant between leaves in the HALFSHADE and SUN treatments ($F_{(2,21)}$ = 7.908, $P < 0.01$), where chlorophyll levels were highest in the leaves exposed to full sun. The Chl *a*/*b* ratio was significantly higher in SUN leaves than those grown in shaded conditions ($F_{(2,21)}$ = 95.84, $P < 0.001$). In addition, per leaf area, SUN leaves also possessed significantly greater pools of both β-carotene ($F_{(2,21)}$ =21.61, $P < 0.001$), and lutein ($F_{(2,21)}$ =13.03, $P < 0.001$) than SHADE leaves, whereas leaves in both SUN and SHADE treatments exhibited similar concentrations of neoxanthin. These findings were largely mirrored when pigments were expressed on

a chlorophyll basis (Table 2). The most notable differences between the leaves in the SUN and SHADE treatments were the greater levels of β-carotene, and particularly the greater concentration of xanthophyll cycle pigments, in the SUN leaves. Consequently, the increase in the sum of all carotenoids in leaves from the SUN treatment primarily reflected increases in these two pigment pools.

Isoprene emissions ($F_{(2,21)} = 12.29$, $P < 0.001$) and photosynthetic assimilation rates ($F_{(2,21)} = 23.05$, $P < 0.001$) were highest in leaves from the SUN treatment, compared to those acclimatised to the SHADE or HALFSHADE treatments (Table 2), whereas PRI of SUN leaves was significantly lower than for leaves in either of the other two treatments ($F_{(2,21)} = 34.17$, $P < 0.001$). Lowest values of EPS were observed in SHADE leaves ($F_{(2,21)} = 4.07$, $P < 0.05$).

Relationships between isoprene emissions, phytopigments and PRI

Isoprene emissions were significantly positively related to carotenoid and chlorophyll pigments per leaf area, in response to differences in the light acclimatisation treatments (Table 3; Fig. 1). Total carotenoid concentration (Fig. 1a), β-carotene and lutein were the carotenoids most strongly correlated with isoprene emissions ($r = 0.8$; $P < 0.0001$). Isoprene emissions were also well correlated with xanthophyll cycle pigment concentration ($r = 0.75$, $P < 0.0001$; Fig. 1b) but not with the epoxidation state of these pigments (EPS; Table 3). When carotenoid concentrations were expressed on a chlorophyll basis, as opposed to per leaf area, the relationship with isoprene emissions was significantly weaker (Table 3). Isoprene emissions were only moderately correlated with the photosynthetic assimilation rate ($r = 0.60$ $P < 0.01$; Fig. 1c).

PRI was most strongly correlated with the size of the xanthophyll cycle pigment pool ($r = -0.82$, $P < 0.0001$; Fig. 2a) and was also strongly correlated with carotenoid concentration (Fig. 2b). The strength of the correlations was similar regardless of whether carotenoids or the xanthophyll pigment concentration was expressed per leaf area or on a chlorophyll basis (Table 3). PRI was also moderately well correlated with isoprene emissions ($r = -0.66$; Fig. 2c) even though PRI and isoprene emissions were found to correlate best with different biochemical variables (Table 3). EPS was not significantly correlated with PRI ($r = -0.1$, $P > 0.05$; Table 3).

Discussion

Effect of sun, shade and half-shade acclimation on leaf chemistry, physiology, isoprene emissions and spectral reflectance

Phytopigments, isoprene emissions, photosynthetic assimilation rates and recorded PRI values of willow leaves acclimatised to different illumination conditions are similar to those observed in other studies and across a range of species.

The higher Chl *a*/*b* ratio for leaves acclimatised to a higher growth irradiance reported in the current study, agree with those reported previously (e.g. Dale and Causton, 1992; Niinemets, 2007) and suggests that leaves acclimatised to shady conditions have greater levels of Chl *b*, and a larger antenna size than sun-exposed leaves, both of which help shaded leaves gather more light (Hallik et al., 2012). When plants experience a large range of light availabilities, there should also be a strong relationship between leaf area-based total carotenoid concentrations and growth irradiance or leaf mass area (LMA; Hallik et al., 2012). The results from our study indicate that the effect of light regime on total carotenoid concentration was significant, with larger total carotenoid pools observed in plants adapted to full sunlight. These findings are similar to a number of other studies that have reported higher carotenoid concentrations in plants transferred from shade to sun conditions, than in plants remaining shaded (Porcar-Castell et al., 2009). Differences in carotenoid concentrations have also been reported along vertical light gradients within natural canopies. For example, Gamon and Berry (2012) observed larger carotenoid concentrations (relative to chlorophyll) in leaves of three conifer species (*Tsuga heterophylla, Pinus ponderosa and Pinus banksiana*), which were exposed to full sun at the top of the canopy, than those located further down in the shade. Similarly, Hallik et al. (2012) also reported an increase in carotenoid concentration along an increasing vertical light availability gradient in natural canopies of two herbaceous species (*Inula salicina, Centaurea jacea*) and two woody species (*Populus tremula, Tilia cordata*).

Acclimation to low light tends to enhance the pools of light-harvesting carotenoids (lutein and its precursor α-carotene; Hallik et al., 2012), which improves light harvesting in deep shade (Krause et al., 2001; Matsubara et al., 2009). Our results show no difference in lutein concentration in *S. viminalis* leaves based on leaf dry mass between shade and sun treatments (data not shown) and slightly higher (9%) lutein concentration (on a chlorophyll basis) in sun-adapted plants. Demmig-Adams (1998) also observed a small (5%) increase in lutein (on a chlorophyll basis) in some sun-exposed leaves compared to shaded leaves. Leaf acclimation to high light tends to increase the pool of carotenoids associated with the efficiency of photosystem I (PSI) and II (PSII), and photoprotection (i.e. β-carotene, its derivatives and the xanthophyll pigments, Z, A and V; Hallik et al., 2012). In our study, β-carotene concentrations in *S. viminalis* leaves, were indeed highest in sun-adapted plants (leaf area and chlorophyll basis). Light-dependent conversion between V, A and Z plays a central role in photoprotection, dissipating excess light energy as heat (Demmig-Adams and Adams, 2006; Muller et al., 2001). Our results show a significantly greater VAZ concentration in *S. viminalis* leaves grown in

full sun conditions compared to shade-adapted leaves (leaf area and chlorophyll basis). Our findings are consistent with other studies, which suggest that sun-exposed leaves invest more in photoprotection relative to those growing in the shade (e.g. Demmig-Adams, 1998; Filella et al., 2009; Gamon and Berry, 2012; Porcar-Castell et al., 2009). As expected, there was a significant effect of shading on isoprene emissions from *S. viminalis*. The higher isoprene emissions observed for plants in full sun compared to those growing in the shade agrees with several other published results (e.g. Harley et al., 1996; Sharkey et al., 1996). Differences in isoprene emissions between sun and shaded leaves can, in part, be attributed to differences in the biochemical and/or physiological properties that influence emission potential. For example due to differences in the proportion of photosynthate allocated to isoprene emissions (Litvak et al., 1996).

PRI values were lower in leaves that were acclimated to full sunlight than those leaves that were subject to either of the shaded treatments (Table 2). PRI was also strongly correlated with total carotenoids and specifically the size of the xanthophyll cycle pigment pool, but not with EPS. These findings are consistent with previous reports that leaf-level PRI, over seasonal timescales, is strongly influenced by constitutive changes in photoprotective pigment concentrations (Gamon and Berry, 2012; Porcar-Castell et al., 2012; Stylinski et al., 2002; Wong and Gamon, 2014).

Relationships between leaf chemistry, physiology, isoprene emissions and spectral reflectance

Our results show a significant positive correlation between isoprene emissions and photosynthesis (Table 3). Similar, correlations between isoprene emissions and photosynthetic light response, has been reported in a range of species (e.g. Kuhn et al., 2004a; Kuhn et al., 2004b; Litvak et al., 1996), suggesting a close relationship between isoprene biosynthesis and carbon (Litvak et al., 1996; Sharkey and Singsaas, 1995). However, our results also show that photosynthesis explains only ~36 % of the variability in isoprene emissions (Fig. 1). Differences in leaf temperature and variations in CO_2 concentration in the leaf cuvette air may contribute to some of the variability. It is possible that some of the observed variability in isoprene emissions may be due to different leaf densities, and perhaps to emission samples being taken at different times of the day with a possible underlying circadian effect on the emissions (Litvak et al., 1996; Loivamaki et al., 2007; Wilkinson et al., 2006), though this has not been demonstrated for *Salix* species. Furthermore, 10-30 % of isoprene production is not directly linked to photosynthesis, but is associated with older carbon sources (Unger et al., 2013). The relationship between photosynthesis and isoprene emissions may also break down under a range of different environmental conditions (see *Introduction*). Consequently, isoprene emissions are not expected to correlate strongly with photosynthesis rate in all situations and conditions (Sanadze, 2004).

As anticipated, dark adaptation of the leaves prior to commencement of the experiment resulted in a non-significant correlation between EPS and isoprene emissions, indicating that short-term facultative changes in the xanthophyll cycle pigment pool (i.e. pigment conversions) were not related to isoprene emissions in our study.

Our results show that isoprene emissions from *S. viminalis* at standard conditions were significantly correlated with total carotenoid concentration, as well as β-carotene, lutein and total xanthophyll pigment concentration. The correlations between carotenoid concentration and isoprene emissions observed in our study could be due to biochemical or functional (anti-oxidant) relationships, or a combination of both. This supports the "Opportunistic hypothesis" (Owen and Peñuelas, 2005) and also concurs with previous studies that report relationships between volatile isoprenoid emission potential and carotenoid pools for light-dependent monoterpene and isoprene emissions from a range of different species (Owen and Peñuelas, 2005; Porcar-Castell et al., 2009).

PRI values were significantly correlated with isoprene emissions (Table 3), which agrees with the results reported by Peñuelas *et al.* (2013). However, the strength of the correlation reported in our study is slightly lower than those reported by Peñuelas *et al.* (2013) for *Populas nigra,* which may in part be due to the narrower range of isoprene emissions produced by *S. vimarlix*, which was approximately half of that observed by Peñuelas *et al.* (2013).

The lack of correlation between PRI and EPS indicates that under the conditions of this experiment, short term facultative adjustments in the epoxidation state of the xanthophyll cycle pigments did not influence PRI. Since isoprene emissions were also strongly correlated with total carotenoid concentrations, and to a slightly lesser extent the size of the xanthophyll cycle pigment pool, but not with EPS; our results strongly support the hypothesis that at longer time scales (weeks to months), the relationship between isoprene emissions and the PRI signal is influenced by constitutive adjustments in carotenoid concentration.

Our results complement those of Peñuelas *et al.* (2013) who suggested that isoprene-PRI relationships under naturally varying illumination conditions were a function of changes in LUE, and thus at least in part, thought to be associated with short-term facultative xanthophyll cycle pigment conversions in response to irradiance. However, we also show that at longer timescales the isoprene-PRI relationship is also likely to be driven by constitutive adjustments in the size of carotenoid pigment pools.

If PRI is to be used as an estimator of isoprenoid emissions or incorporated into isoprenoid emission models, then knowledge of how isoprene emissions are influenced by both the longer term effects of growth irradiance on carotenoid pigment concentrations observed in our study, and the dynamic adjustments of xanthophyll cycle pigments suggested by Peñuelas *et al.* (2013), have important implications for interpreting the PRI-isoprene relationship. This may be especially true where facultative and constitutive changes in pigments are out of phase (Gamon and Berry, 2012; Sims et al., 2006; Wong and Gamon, 2014). Consequently, further work is needed to isolate and understand the relative influence of short term facultative and longer term constitutive changes in carotenoid pigment pools on the relationship between isoprenoid emissions and PRI, across a wider range of species and in field conditions.

Author contributions

AH and SMO conceived, designed and performed the experiment. DS and MGP generated the pigment data. AH and SMO analysed the data and wrote the manuscript.

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Figure Legends

Fig. 1 Correlations between isoprene emissions and a) total carotenoids, b) xanthophyll cycle pigment pool (VAZ) and c) photosynthetic assimilation rates. Lines are fitted for reasons of clarity only. ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Fig. 2 Correlations between the photochemical reflectance index (PRI) and a) xanthophyll cycle pigment pool (VAZ), b) total carotenoids and c) isoprene emissions. Lines are fitted for reasons of clarity only. ns = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Table 1 Analytical gradient protocol for elution of pigments using HPLC

Time (mins)	% Solvent A	% Solvent B
0	95	5
22	30	70
35	95	5
36	0	100
42.5	0	100
43	95	5
50	95	5

Solvent A - 70% MeOH plus 30% 28mM Tetra butyl ammonium acetate TBAA; solvent B - methanol

Table 2 Differences in pigment composition, isoprene emissions, photosynthetic assimilation rates, epoxidation state of the xanthophyll cycle pigments, and the photochemical reflectance index from pooled data for SHADE, HALFSHADE and SUN treatment leaves.

	SHADE		HALFSHADE		SUN		SUN, % of SHADE
	Mean	SD	Mean	SD	Mean	SD	
Chl *a*/*b* ratio	3.5 *a*	0.1	3.6 *b*	0.1	4.0 *c*	0.1	16.0 %
Pigment concentration on a leaf area basis							
Chl (μmol m^{-2})	1171.4 *ab*	157.8	1038.4 *a*	113.5	1363.3 *b*	220.9	-
Car (μmol m^{-2})	274.9 *a*	36.8	264.5 *a*	24.8	409.0 *b*	52.2	48.8 %
VAZ (μmol m^{-2})	45.1 *a*	5.9	51.8 *a*	4.5	112.8 *b*	14.7	150.1 %
β- Carotene (μmol m^{-2})	71.8 *a*	11.8	64.6 *a*	7.5	100.5 *b*	14.7	39.9 %
Lutein (μmol m^{-2})	116.9 *a*	14.6	110.9 *a*	10.2	147.8 *b*	19.7	26.4 %
Neoxanthin (μmol m^{-2})	41.1 *ab*	5.8	37.1 *b*	4.7	47.8 *a*	7.7	-
Pigment concentration on a chlorophyll basis							
Car Chl^{-1} (mmol mol^{-1})	234.9 *a*	9.1	255.4 *b*	14.8	301.9 *c*	20.4	28.5 %
VAZ Chl^{-1} (mmol mol^{-1})	38.6 *a*	3.2	50.2 *b*	4.4	83.7 *c*	10.8	116.6 %
β-Carotene Chl^{-1} (mmol mol^{-1})	61.2 *a*	3.7	62.3 *a*	4.3	73.9 *b*	2.8	20.9 %
Lutein Chl^{-1} (mmol mol^{-1})	100.0 *a*	4.2	107.1 *b*	6.7	109.0 *b*	5.6	9.0 %
Neoxanthin Chl^{-1} (mmol mol^{-1})	35.1 *a*	2.1	35.8 *a*	2.8	35.3 *a*	4.0	-
Isoprene emission potential (nmol m^{-2} s^{-1})	2.7 *a*	2.6	0.8 *a*	1.0	7.3 *b*	3.6	172.6 %
Photosynthetic assimilation rate (μmol m^{-2} s^{-1})	7.7 *a*	1.4	9.8 *b*	0.9	12.2 *c*	1.5	58.0 %
PRI	0.05 *a*	0.01	0.05 *a*	0.00	0.03 *b*	0.01	-45.0 %
EPS	0.61 *a*	0.07	0.77 *b*	0.16	0.75 *ab*	0.13	-

The Tukey test compared differences between treatments at the 5% level of significance ($P < 0.05$). Same letters indicate mean values are not significantly different in the horizontal direction. Percentage differences (SUN, % of SHADE) are not calculated when there is no statistically significant difference between SHADE and SUN treatments. Abbreviations: Chl, sum of Chl *a* + *b*; Car, sum of all carotenoids; VAZ, sum of violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z); PRI, photochemical reflectance index; EPS: epoxidation state of xanthophyll cycle pigments.

Table 3 Correlation matrix showing the Pearson's correlation coefficient (*r*) between variables.

	PRI	EPS	Isoprene	A	Chl	Car	VAZ	βC	L	N	Car Chl^{-1}	VAZ Chl^{-1}
PRI	1											
EPS	-0.10	1										
Isoprene	-0.66***	0.11	1									
A	-0.50*	0.33	0.60**	1								
Chl	-0.47*	0.02	0.83****	0.52**	1							
Car	-0.75****	0.23	0.84****	0.71***	0.86****	1						
VAZ	-0.82****	0.28	0.75****	0.77****	0.67***	0.94****	1					
βC	-0.70****	0.16	0.89****	0.66***	0.98****	0.98****	0.86****	1				
L	-0.64***	0.16	0.81****	0.63*	0.97****	0.97****	0.85****	0.97****	1			
N	-0.46*	0.29	0.73***	0.36	0.84****	0.83****	0.65***	0.87****	0.84****	1		
Car Chl^{-1}	-0.77****	0.42*	0.45*	0.62**	0.20	0.67***	0.83****	0.53**	0.51*	0.35	1	
VAZ Chl^{-1}	-0.80****	0.36	0.53**	0.72***	0.32	0.74****	0.91****	0.60**	0.59**	0.38	0.97****	1

Abbreviations: PRI, photochemical reflectance index; EPS, epoxidation state of the xanthophyll cycle pigments; Isoprene, isoprene emissions ((nmol m^{-2} s^{-1}); A, photosynthetic assimilation rate (μmol m^{-2} s^{-1}) ; Chl, sum of chl *a* + *b* (μmol m^{-2}); Car, sum off all carotenoids (μmol m^{-2}); VAZ, sum of violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z) (μmol m^{-2}); βC, β-carotene (μmol m^{-2}); L, lutein (μmol m^{-2}) ; N, neoxanthin (μmol m^{-2}); Car Chl^{-1} , total carotenoids expressed on a chlorophyll basis (mmol mol^{-1}); VAZ Chl^{-1}, sum of violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z) expressed on a chlorophyll basis (mmol mol^{-1}); Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

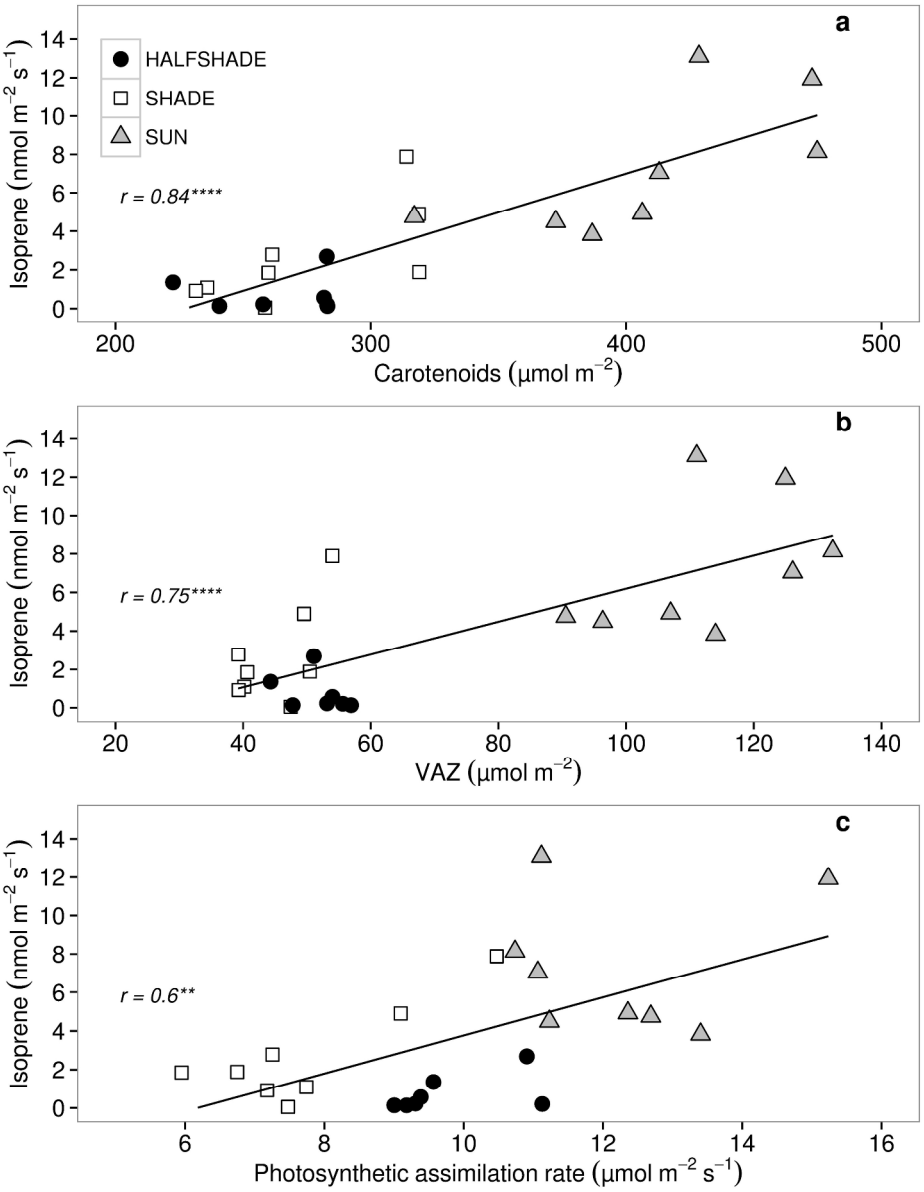

Fig. 1 Correlations between isoprene emissions and a) total carotenoids, b) xanthophyll cycle pigment pool (VAZ) and c) photosynthetic assimilation rates. Lines are fitted for reasons of clarity only. ns = not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.
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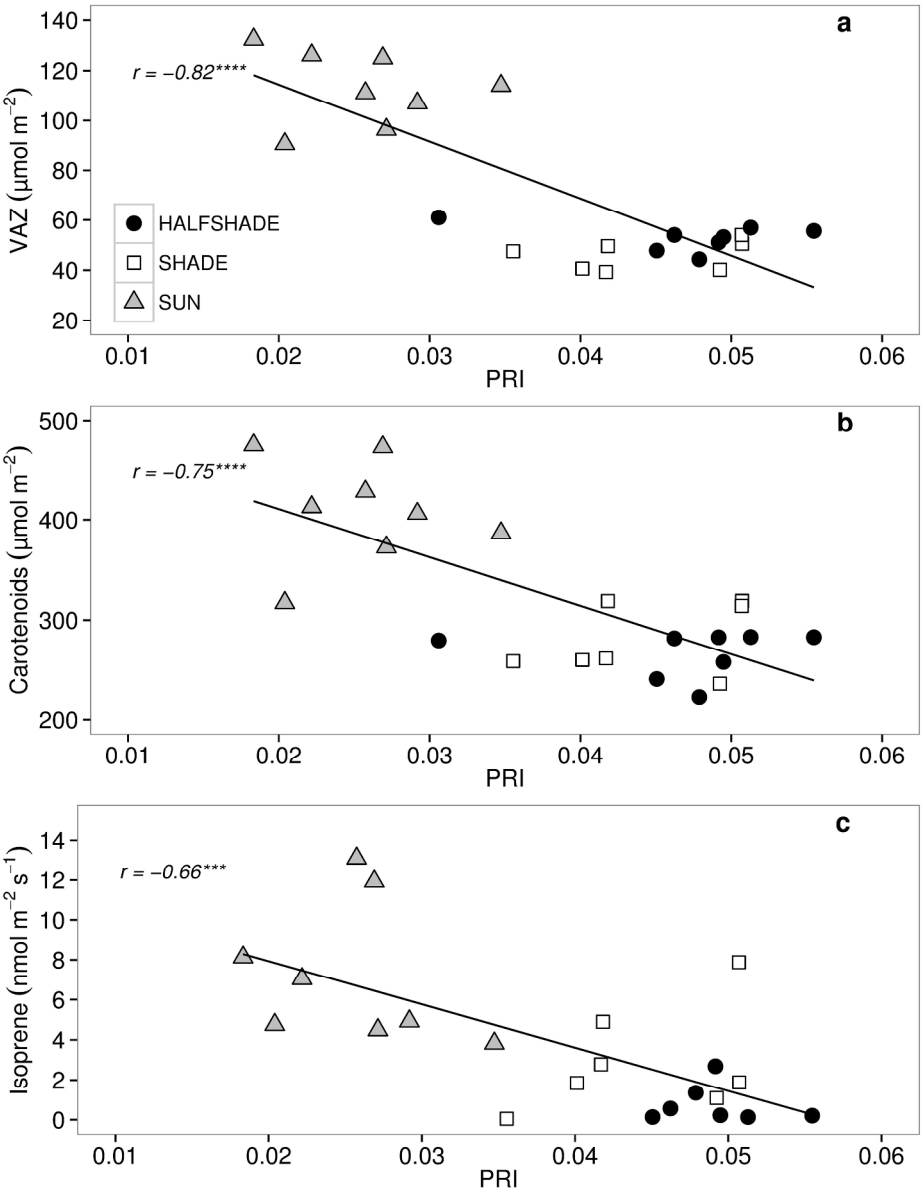

Fig. 2 Correlations between the photochemical reflectance index (PRI) and a) xanthophyll cycle pigment pool (VAZ), b) total carotenoids and c) isoprene emissions. Lines are fitted for reasons of clarity only. ns = not significant; * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.
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