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Original article

In situ optical properties of foliar flavonoids: Implication for non-destructive estimation of flavonoid content

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ABSTRACT

Flavonoids are a ubiquitous multifunctional group of phenolics of paramount importance for the terrestrial plants involved in protection from biotic and abiotic stresses, color and chemical signaling and other functions. Deciphering of *in situ* absorption of foliar Flv is important but was thought to be impossible due to a strong overlap with other pigments, complex *in situ* chemistry of Flv and sophisticated leaf optics.

We deduced *in situ* absorbance of foliar Flv and introduced a concept of specific absorbance spectrum indicative of each pigment group contribution to light absorption and provided a rationale for the choice of spectral bands for non-destructive assessment of Flv in leaves with variable content of other pigments including anthocyanins.

Only a narrow band 400–430 nm was suitable for Flv assessment, however the effect of other pigments remained substantial, so subtraction of their contribution was necessary. The devised leaf absorbance-based algorithm allowed estimating Flv with error below 21%.

Absorption by Flv in plant tissues might extend into the blue and can be commensurate to that of chlorophylls and carotenoids. The potential capacity of Flv to shield the cell *in situ* from the visible light might be essential for assessments of high light stress tolerance of plants.

1. Introduction

Flavonoids (Flv) constitute a large and diverse group of phenolic compounds ubiquitous in higher plants in which they fulfill a large number of functions (Agati et al., 2013; Harborne and Williams, 2000; Markham, 1989). Participating in the protection from biotic and abiotic stresses is reportedly among the most important functions of Flv along with color and chemical signaling and other auxiliary roles (Agati et al., 2013; Harborne and Williams, 2000). In particular, Flv constitute the first-line defense against photodamage by UV (Day et al., 1994; Havaux and Kloppstech, 2001; Kolb and Pfundel, 2005; Liakoura et al., 2003; Mazza et al., 2000). Accumulation of Flv was documented among the primary responses to diverse stresses, mostly to high solar and/or UV irradiation (Kolb et al., 2001; Tattini et al., 2004). Those include inhibition of photosynthesis and growth, a decrease in yield due to direct or indirect primary effects on membranous systems, proteins and nucleic acids as well as phytopathogen and grazer attacks and wounding (Ballare et al., 2011; Jansen et al., 1998; Rozema et al., 1997). The protective effects of Flv are implemented via optical screening mechanism augmented by a prominent antioxidant capacity of these compounds (Agati et al., 2012; Agati et al., 2013) or, in the case of pathogen defense, by antifungal and bactericide effects (Harborne and Williams, 2000; Markham, 1989; Treutter, 2006). From the practical standpoint, Flv are also important as potent antioxidants (Rice-Evans et al., 1997; Russo et al., 2000; Tournaire et al., 1993), which exert multifaceted beneficial effects on human health providing protection from cardiovascular and oncology diseases (Van der Sluis et al., 1997; Williamson et al., 2000).

In line with their functions, Flv are concentrated in the surface structures and tissues of plants, mainly in cuticle, often in concentrations considerably higher than concentrations of photosynthetic pigments, chlorophylls (Chl) and carotenoids (Car) (Krauss et al., 1997; Solovchenko and Merzlyak, 2003) and epidermis (Day et al., 1994; Mazza et al., 2000; Tevini et al., 1991). Flv are precursors of

Abbreviations: AnC, , anthocyanin(s); [AnC], , anthocyanin content; Car, , carotenoid(s); [Car], , carotenoid content; Chl, , chlorophyll(s); [Chl], , chlorophyll content; Flv, , flavonoid(s); [Flv], , flavonoid content

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anthocyanins (AnC), another widespread group of phenolic pigments (Hughes, 2011; Strack and Wray, 1989; Tattini et al., 2017). Accumulation of Flv affects profoundly the optical properties of leaves (Day et al., 1994; Pfiindel et al., 2006) and fruit (Merzlyak et al., 2005) accommodating these pigments vastly increasing absorption (and decreasing reflectance) of light in the blue-violet part of the visible spectrum.

Numerous studies on mechanisms of Flv roles in UV protection in plants were focused on optical properties as well as phenolic content and composition of a detached superficial layer of a leaf consisting of epidermal cells and cuticle (Barnes et al., 1996; Kolb and Pfundel, 2005; Markstädter et al., 2001). Since the estimation of Flv contents and its trends provides important clues about potential of a plant organism for acclimation to and cope with a wide array of stresses, resistance to phytopathogens and health beneficial value (Agati et al., 2013; Harborne and Williams, 2000; Markham, 1989), a number of attempts have been made to develop an approach for non-invasive assay of Flv *in situ*. These attempts involved mostly the analysis of the fluorescence of Chl excited in the UV or red regions (Agati et al., 2016; Cerovic et al., 2002; Lenk et al., 2007).

Recently, a conceptual approach relating remotely sensed optical properties and pigment content was developed and applied successfully for non-destructive estimation of Chl, Car, and AnC in higher plant leaves (Gitelson et al., 2003; Gitelson et al., 2006). However, this approach was not tested for estimation of Flv in leaves. One can anticipate that the main obstacles for in situ quantification of Flv are constituted by strong, overlapping, and variable contributions by Chl, Car and other phenolic compounds e.g., AnC and phenylpropanoids (Day et al., 1994; Solovchenko and Merzlyak, 2003). Another interference is raised by the variability in leaf structure and thickness or, in effect, by its scattering properties (Féret et al., 2017; Gitelson et al., 2003).

In this study we attempted (as far as we know, for the first time) to investigate quantitatively the *in situ* spectral properties of Flv in leaves in more detail and examined the applicability of non-destructive Flv estimation using absorbance spectra.

2. Methods

Two plant species, Common cotoneaster (*Cotoneaster integerrimus* Medik.), and Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.) characterized by a widely varying content of pigments (Fig. 1), especially of AnC and Flv (El-Mousallamy et al., 2000; Gitelson et al., 2001) served as model species for this study. Healthy and homogeneously colored leaves were randomly collected according to their coloration in a park at Moscow State University in 2016. AnC were abundant during spring and autumn in sunlit leaves. In these species, AnC were predominantly in the vacuoles of the cell layer of palisade parenchyma, see (Merzlyak et al., 2008) for more details.

The procedure allowing simultaneous quantification of total Chl, Car, AnC, and Flv in an extract from leaf zone used for transmittance measurements was employed essentially as described in Solovchenko et al. (2001). Leaf disks (total area of 3.8 cm^2) were ground in chloroform–methanol (2:1, vol/vol) in the presence of MgO. After completion of extraction, homogenates were filtered through a paper filter, and distilled water (1/5 of total extract volume) was added. Then extracts were centrifuged at 3000*g* for 10 min until phase separation.

Total Chl and Car concentrations were quantified spectrophotometrically in lower (chloroform) phase using coefficients reported by Wellburn (1994). The upper (water–methanol) phase was used for assay of total Flv, which were quantified spectrophotometrically using the band around 358 nm where Flv exert the dominant contribution to the absorption and molar absorption coefficient ε_{358} = 25.4 $mM^{-1}\,cm^{-1}$ determined for rutin in 80% aqueous methanol. After determination of Flv the water–methanol phase was acidified with HCl (final concentration of HCl = 0.1%) and used for quantification of anthocyanins by measuring absorbance at 530 nm; absorption coefficient of 30 $mM^{-1}\,cm^{-1}$ (Strack and Wray, 1989) was accepted. Flv (in equivalent amounts of rutin) as well as other pigment content were expressed relative to leaf surface area.

Leaf transmittance (T) spectra were recorded with a 150–20 Hitachi spectrophotometer equipped with an integrating sphere against barium sulphate as a standard. The spectra were recorded at 2-nm sampling intervals in 350–800 nm range. Absorbance (α) was calculated via transmittance as $\alpha = -\ln T$.

3. Results

Data sets used for investigation of Flv optical properties contained 24 *P. quinquefolia* and 10*C.integerrimus* leaves with widely variable pigment contents and composition (Fig. 1). Notably, large amounts of Flv were present in all leaves collected, including the leaves with minute amounts of Chl, Car and AnC. To understand how leaf absorbance is affected by contents of different pigments including Flv, absorbance spectra were analyzed; the spectra of four *Parthenocissus* leaves with widely varying pigment contents are shown in Fig. 2. In the range 600–750 nm Chl *a* and *b* govern absorbance that peaks *in situ* around 675 nm (see also Gitelson et al., 2003). In the range 500–600 nm, AnC greatly affect absorbance; in red leaves, it almost reaches the level of absorbance in the red range or may even surpass it in the case of high AnC/Chl molar ratios. Absorbance in the green range is also affected by Chl although to a less degree than by AnC (Gitelson et al., 2001). Since both pigments, AnC and Chl, play role in absorbance in the green, the fractions of [Chl] and [AnC] define absorbance value in this spectral range.

In the blue range (400–500 nm) all four pigments (Chl, Car, AnC, and Flv) affects the leaf absorbance (Fig. 2). However, there is no specific spectral feature of each pigment group although their contributions might vary considerably. The highest contribution of the tailing absorption of Flv which can be commensurate to or even higher than that of Chl and Car was recorded in the green and yellow leaves. Notably, a higher [Flv] of the yellow leaf than that of the green leaf (despite higher [Chl] of the green leaf) clearly manifested itself as a higher absorbance at 400–420 nm. The leaves with a high [AnC] displayed a pronounced trough in the range 420–460 nm caused by decreasing AnC absorbance on the background of the Flv absorbance that increased toward shorter wavelengths. In leaves with either conservative or invariable [Chl], increase in [Flv] exerts dominating contribution causing increase in absorbance in the shortwave blue range 400–430 nm (Fig. S1). Thus, the effect of [Flv] on absorbance could be seen more clearly only in leaves with variable [Flv] and quite narrow variation of [Chl] and [Car] and in leaves with even [Chl] (Fig. S1), allowing to locate a spectral region between 400 and 430 nm where Flv appears to be main although not sole chromophore affecting leaf absorbance.

To quantify the effect of each pigment content, [p], on absorbance, α, relationships α vs. [p] at each wavelength, λ, for each pigment were established. The slopes of these relationships at particular wavelength are indicative of sensitivity of absorbance a_λ to each pigment content (Fig. 3A). As we emphasized above, the studied leaves differed widely in their pigment content and composition — [Car]/[Chl] ≈ 0.3 and [Flv] was considerably higher than [Chl] — [Flv]/[Chl] > 10 for 17 of 24 leaves and [Flv]/[Chl] > 5 for other 7 leaves. Therefore, to compare sensitivity of absorbance to each type of pigment, it is necessarily to adjust the slopes to average pigment content. Thus, we multiply the slope of α to [Flv] relationship to average ratio of [Flv]/[Chl] = 10 and the slope of α to [Car] relationship to average ratio of [Car]/[Chl] = 0.3. Slopes of α to [Pigment] presented in Fig. 3a took into account the relative pigment contents ratios and represent a sensitivity of α to each pigment.

In the range 530–600 nm, absorbance was maximally sensitive to [AnC] and sensitivity to [Chl] and [Car] was much smaller. Absorbance was sensitive to content of all four pigment groups in the range