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ORIGINAL ARTICLE

Lettuce flavonoids screening and phenotyping by chlorophyll fluorescence excitation ratio

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Abstract

Main conclusion **Environmentally induced variation and the genotypic differences in flavonoid and phenolic content in lettuce can be reliably detected using the appropriate parameters derived from the records of rapid non-invasive fluorescence technique.**

The chlorophyll fluorescence excitation ratio method was designed as a rapid and non-invasive tool to estimate the content of UV-absorbing phenolic compounds in plants. Using this technique, we have assessed the dynamics of accumulation of flavonoids related to developmental changes and environmental effects. Moreover, we have tested appropriateness of the method to identify the genotypic differences and fluctuations in total phenolics and flavonoid content in lettuce. Six green and two red genotypes of lettuce (*Lactuca sativa* L.) grown in pots were exposed to two different environments for 50 days: direct sunlight (UV-exposed) and greenhouse conditions (low UV). The indices based on the measurements of chlorophyll fluorescence after red, green and UV excitation indicated increase of the content of UV-absorbing compounds and anthocyanins in the epidermis of lettuce leaves. In similar, the biochemical analyses performed at the end of the experiment confirmed significantly higher total phenolic and flavonoid content in lettuce plants exposed to direct sun compared to greenhouse conditions and in red compared to green genotypes. As the correlation between the standard fluorescence indices and the biochemical records was negatively influenced by the presence of red genotypes, we proposed the use of a new parameter named Modified Flavonoid Index (MFI) taking into an account both absorbance changes due to flavonol and anthocyanin content, for which the correlation with flavonoid and phenolic content was relatively good. Thus, our results confirmed that the fluorescence excitation ratio method is useful for identifying the major differences in phenolic and flavonoid content in lettuce plants and it can be used for high-throughput prescreening and phenotyping of leafy vegetables in research and breeding applications towards improvement of vegetable health effects.

Keywords Phenolic · Flavonoids · Chlorophyll fluorescence · Lettuce · UV radiation · Phenotyping

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Abbreviations

ANTH	Fluorescence-based index for estimation of anthocyanin content in plant tissues
Anth	Anthocyanins
DW	Dry weight
FER	Fluorescence excitation ratio
FLAV	Fluorescence-based index for estimation of flavonol content in plant tissues
Flav	Flavonoids
$FLAV_C$	Corrected fluorescence-based index for estimation of flavonol content
FRF	Far-red fluorescence, fluorescence with wavelength ~730 nm
FRF_G	Far-red fluorescence emitted after excitation by green light
FRF_R	Far-red fluorescence emitted after excitation by red light
FRF_{UV}	Far-red fluorescence emitted after excitation by UV
MFI	Modified flavonoid index

Introduction

Lettuce is an important agricultural commodity worldwide, providing an important source of different nutritionally valuable substances. The potential beneficial effect of lettuce can be enhanced by the antioxidants such as polyphenols, which can play an important role in preventive nutrition (Nicolle et al. 2004). To increase the quality and health effects of this crop, efficient ways to utilize better a huge germplasm of this plant will be needed.

Plant phenolic compounds, also denoted polyphenols, are plant secondary metabolites, defined as compounds possessing one or more aromatic rings bearing hydroxyl substituent(s) (Parr and Bolwell 2000). Phenolic compounds can be generally divided into two categories, flavonoids and non-flavonoid polyphenols. Non-flavonoids and phenolic acids can be further branched into two main types, benzoic acid and cinnamic acid derivatives (Chandrasekara and Shahidi 2010). Flavonoids, the target class and the most common and widely distributed group of polyphenols, may be divided into different subclasses according to the oxidation state of the heterocyclic ring: anthocyanins, flavonols, flavans, flavanols, flavones, and isoflavones (Scalbert and Williamson 2000; Kumar and Pandey 2013). Flavonoids occur virtually in all plant parts, particularly the photosynthesizing plant cells (Scalbert and Williamson 2000). As a dietary component, flavonoids have been suggested to have health-promoting properties due to their high antioxidant capacity both in vivo and in vitro systems (Cook and Samman 1996). In plants, these compounds usually appear as a response to exposure to ultraviolet radiation and the accumulation of UV-B-absorbing phenolic compounds is generally described as a key element in acclimation to UV-B exposure (Bassman 2004); however, their increase was observed also in the presence of high irradiation and absence of UV (Agati et al. 2011). Flavonoids broadly have a role in many facets of plant physiology. They can play a role of antioxidants or oxygen scavengers (Nishiyama et al. 2001, 2006; Murata et al. 2007; Weidner et al. 2007; Amarowicz et al. 2010). Hence, the flavonoids could also play a considerable role in plant responses to environmental factors, in particular during biotic and abiotic stresses (Weidner et al. 2001; Nishiyama et al. 2001, 2006; Wróbel et al. 2005; Murata et al. 2007), although the effects are sometimes limited only to short exposure to the stress conditions (Król et al. 2014). Besides that, plants need phenolic compounds for pigmentation, growth, reproduction and resistance to pathogens (Sytar et al. 2012).

HPLC profile of phenolic compounds in fresh lettuce has been identified, showing a high contribution of protocatechic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid and phloridzin (Altunkaya and Gökmen 2009). In addition, a significant, but variable content of quercetin content was obtained by HPLC in different varieties of lettuce plants (Crozier et al. 1997). Quercetin and cyanidin glycosides were identified as the main flavonoid components in the lettuce and their concentration in lettuce was found to be highly sensitive to the transmission of UV light (Garcia-Macias et al. 2007). Anyway, it must be taken into an account that significant differences in phenolics content and related antioxidant activity between red and green varieties of lettuce were observed (Ozgen and Sekerci 2011). In red color genotypes of lettuce, the cyanidin, a particular type of anthocyanin, is the main (but not the only) constituent responsible for the red coloration (Garcia-Macias et al. 2007). Red lettuce varieties have been characterized by a higher content of hydroxycinnamic acids, flavones, flavonols and anthocyanins compared to the green varieties of lettuce plants (Llorach et al. 2008).

Typically, in addition to a high variation in the flavonoid content among cultivars, the effect of growth conditions is also significant (Nicolle et al. 2004). From the external factors, the flavonoid content strongly depends on the intensity of incident UV radiation (Zhang and Björn 2009). It was found that light-responsive flavonoids containing dihydroxy B-ring-substituted structures are the most effective in preventing the generation and scavenging reactive oxygen species (Agati and Tattini 2010; Agati et al. 2011). Thanks to genotypic and environmentally induced variability in flavonoid content, it can be expected that the screening and exploitation of genetic resources in breeding can lead to increase of flavonoid content in new genotypes. The success of screening process depends strongly on the number of genotypes tested. However, the process of analyzing the phenolic content using

conventional biochemical methods can be rather laborious and expensive, especially if the interaction of genotype x environment is studied. The possible solution is the application of fast pre-screening methods. Cerovic et al. (2002) have introduced an approach of estimate of UV-absorbing compounds based on chlorophyll fluorescence measurements, called fluorescence excitation ratio method (FER method). Different methods of chlorophyll fluorescence analysis are well established in studies of the environmental effects on photosynthetic apparatus (Brestic et al. 2012; Zivcak et al. 2014; Kalaji et al. 2016). Unlike the analysis of photosynthetic traits, the estimation of phenolic content is based on the comparison of fluorescence intensities emitted by the sample after excitation by the monochromatic light with different wavelengths. The measured signal can be called also the multispectrally induced fluorescence (Sytar et al. 2016). As the chlorophylls are located in deeper layers (mesophyll), the excitation light reaching the chloroplasts is attenuated by compounds located in the epidermis. Thus, there is an inverse relationship between the concentration of compounds absorbing particular light wavelength and the intensity of the chlorophyll fluorescence signal (Burchard et al. 2000; Bilger et al. 2001). In this respect, comparison of signals emitted after red and UV excitations can be used for the estimation of UV-absorbing compounds, mostly flavonols (Cerovic et al. 2002), whereas the fluorescence signal emitted after red and green excitations can be used for the estimation of anthocyanins (Agati et al. 2005); signals can be measured simultaneously.

This technique, though not yet widely used, has become more popular due to introduction of commercially available devices in the last decade. It was previously used for estimates of anthocyanins in grapes and other fruits (Ghozlen et al. 2010; Sivankalyani et al. 2016), and leaves of different species (Pfündel et al. 2007; Müller et al. 2013; Bidel et al. 2015), as well as for estimates of flavonoids in medicinal herbs and plants used in human nutrition (Agati et al. 2011; Sytar et al. 2014, 2015) or as an indirect indicator of nitrogen nutrition status (Tremblay et al. 2007; Cerovic et al. 2015). Moreover, in vivo investigation of leaf constitutive flavonols playing a role in the defense of *Vitis vinifera* against downy mildew was also performed (Latouche et al. 2013).

In analogy to previous applications, in our study we tested the usefulness of the technique in research targeted at phenolic compounds in the aboveground parts of lettuce plants. We assessed the ability of the technique to recognize the significant differences in accumulation of phenolic compounds between genotypes and growing conditions differing mostly by the presence of UV radiation in the light spectra. Our results demonstrate that the appropriate parameters can serve to identify the lettuce plants differing in accumulation of flavonoids.

Materials and methods

Plant material and cultivation

In the experiment, we tested eight commercially available lettuce genotypes (*Lactuca sativa* L.), six green varieties of iceberg type (cv. Prazan, cv. Tarzan), buterhead type (cv. Jarny, cv. Cassini), romaine type (cv. Globus), green oak leaf type (cv. Dubacek), as well as two red varieties of red oak leaf type (cv. Redin) and lollo rosso type (cv. Roden). Plants were grown individually in pots with the standard peat substrate (pH 6.5). The early phase of cultivation was carried out in a growth chamber under artificial light (fluorescent tubes Osram Fluora; light intensity 250 µmol photons m^{-2} s^{-1}, photoperiod 10/14 h (light/-dark) at temperature 23/18 °C day/night until the stage of three leaves.

Experimental design and treatments

The experiment was carried out in March and April. After the stage of three leaves, lettuce plants were divided in two groups: a half of lettuce plants was exposed to direct sunlight immediately and natural weather condition in a vegetation cage (variant "Direct sun") and the second group was placed inside behind the clear glass (greenhouse conditions) with a similar light intensity compared to the exterior (variant "Glass"). Throughout the whole experiment, lettuces were regularly irrigated and fertilized using liquid fertilizer with micronutrients. The light conditions in both environments were almost similar. The plants exposed to "Direct Sun" were grown under natural weather conditions. The temperature outdoors were fluctuating (the minimum and maximum daily temperature are presented in Fig. 4d, in "Discussion"). The plants cultivated in greenhouse conditions were grown at a relatively stable temperature, ranging mostly between 18 and 25 °C.

Measurements of fluorescence signals

Measurements of chlorophyll fluorescence under the multiple light spectra excitations were done using the portable optical fluorescence sensor Multiplex-3® (Force-A, France). Multiplex-3® is hand-held battery-operated multi-parametric sensor based on light-emitting-diode excitation and filtered-photodiode detection that is designed to work in the field under daylight. The apparatus has three detector channels and generates four excitations. The sensor has six UV light sources (LED matrices) at 375 nm and it has three, red-blue-green LED-matrices emitting light at 470 nm (blue), 516 nm (green) and 635 nm (red). There are three, synchronized, photodiode

detectors for fluorescence recording: yellow, red and far-red (Ghozlen et al. 2010). In our experiments, we used values of fluorescence measured at 735 nm (FRF) after excitation by red light (635 nm), green light (516 nm) and UV (375 nm).

The parameters for estimation of different groups of phenolic compounds were calculated on the basis of the mutual ratios of fluorescence values at excitations by various colors of light. Based on the Beer–Lambert's law, in analogy to a spectrophotometric method of assessment of leaf absorbance, the parameters were calculated as logarithmic values of the fluorescence ratios.

Analogically to previous studies (Cerovic et al. 2002; Agati et al. 2011), the Flavonoid (FLAV) Index serving as an estimate of UV-absorbing compounds ($\log FER_{R/UV}$), mostly flavonols, was calculated as the natural logarithm of the ratio of the red-light-induced far-red fluorescence (FRF_R) and UV-induced far-red fluorescence (FRF_{UV}):

$$\log FER_{R/UV}(\text{FLAV index}) = \log[FRF_R/(k_{UV} \times FRF_{UV})].$$

Similarly, the *ANTH* Index serving as an estimate of green light absorbing compounds ($\log FER_{R/G}$), mostly red-colored flavonoids, anthocyanins, was calculated as the natural logarithm of the ratio of the red-light induced fluorescence (FRF_R) and green light-induced fluorescence (FRF_G):

$$\log FER_{R/G}(\text{ANTH index}) = \log[FRF_R/(k_G \times FRF_G)].$$

The correction coefficients (constant values) k_{UV} or k_G were added to a measured value to avoid the negative results (as the logarithm of the value lower than 1 is negative). The constants can be specific for each device, especially if the instruments differ in a factory setup (as the devices are typically optimized for particular types of the samples). The values of constants were determined as the minimum values of the ratios (FRF_{UV}/FRF_R or FRF_G/FRF_R) occurring in a database containing a high number (several thousand) of records in different plant species grown in different environments. The same constants have been used in the processing of all the data within an experiment.

The values of indices presented in our paper are different from values of parameters *FLAV* and *ANTH* calculated automatically by the software of the device (due to the addition of the correction constants k_{UV} or k_G); therefore, we will use the abbreviations $\log FER_{R/UV}$ and $\log FER_{R/G}$ instead, and we will use the *FLAV* and *ANTH* as general abbreviations indicating the signal of flavonoids and anthocyanins in the leaf epidermis.

To eliminate the anthocyanin-related signal from the records of $\log FER_{R/UV}$, the approach of Latouche et al. (2013) was adapted. As logFER values are equivalent to absorbance, the two contributions of flavonols and anthocyanins to chlorophyll fluorescence screening at 375 nm can be calculated from the ratio of the excitation coefficients of anthocyanins at 516 and 375 nm. Knowing that flavonols are not absorbing red light nor green light, the $\log FER_{R/G}$ can be used to calculate the pure concentration of anthocyanins (its absorption, its contribution to the chlorophyll screening). From it, the contribution of anthocyanins to the absorbance (screening) at 375 nm can be calculated knowing the extinction coefficient of anthocyanins at 375 nm. Thus, corrected flavonoid parameter, in which contribution of anthocyanins to the signal at 375 nm is subtracted, can be calculated as:

$$FLAV_C = \log FER_{R/UV} - \varepsilon_{375}/\varepsilon_{516} \times \log FER_{R/G},$$

where $\varepsilon_{375}/\varepsilon_{516}$ was calculated as the ratio of extinction coefficient of cyanidin-3-glucoside (the main anthocyanin constituent in lettuce) at 375 and 516 nm, respectively. The value of $\varepsilon_{375}/\varepsilon_{516}$ equal to 0.14 was estimated according to the absorbance profile published by Skaar et al. (2014).

We also proposed a new parameter called Modified Flavonoid Index (MFI) serving as an estimate of total flavonoid content (for justification see the "Discussion" section), calculated as the natural logarithm of the ratio of the red-light induced fluorescence (FRF_R) and green light-induced fluorescence (FRF_G).

$$MFI = \log[2FRF_R/(k_G \times FRF_G + k_{UV} \times FRF_{UV})].$$

The same values of correction constants (k_G, k_{UV}) as for $\log FER_{R/UV}$ and $\log FER_{R/G}$ were used for calculations of MFI.

The measurements of multispectrally induced fluorescence were done in 7–10 days interval during two months. The non-invasive, non-contact measurements were done from the top (top-view) in the central part of the plant, using the large screen; the measured area of a single record was app. 50 cm^2. The duration of one measurement was app. 2 s.

Assessment of total phenolic content

Total phenolic content in the lettuce leaves extracts was determined by the standard spectrophotometric method of Lachman et al. (2003) using Folin–Ciocalteu reagent (Singleton and Rossi 1965). Freeze-dried powdered samples (0.25 g) were extracted for 16–18 h with 20 ml of 80% ethanol. After the time of extraction, a volume of 100 µl of the plant extract was pipetted into 50 ml volumetric flask. 2.5 ml of Folin–Ciocalteau reagent was added to the extract. Then, after 3 min (agitation) 5 ml 20% Na_2CO_3 solution was mixed. After 2 h at 25 °C, the absorbance was measured on the spectrophotometer Jenway UV/Vis. 6405 (Jenway, UK) at wavelength

$\lambda = 765$ nm against the blank. Gallic acid was used as a reference standard for plotting calibration curve. Total phenolic content was expressed as mg kg^{-1} gallic acid equivalent of dry matter. Four samples of each variant were analyzed.

Detection of total flavonoid content

Aluminum chloride colorimetric method of total flavonoid detection was used in four samples of each variant. Quercetin was used to make the calibration curve. Ten milligrams of quercetin was dissolved in 80% ethanol and then diluted to 25, 50 and 100 g ml^{-1}. The diluted standard solutions (0.5 ml) were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Jenway 6405 UV/VIS spectrophotometer (Jenway, UK). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in the blank. Similarly, 0.5 ml of ethanol extracts or 15 flavonoid standard solutions (100 ppm) were reacted with aluminum chloride for determination of flavonoid content as described above.

Data processing and analysis

The statistical significance of differences of these data was evaluated using analysis of variance (ANOVA) using the software STATISTICA 9 (StatSoft Inc.). The results were expressed as mean ± standard errors. The correlation analysis was done for relationships between the parameters of the FER method (predicted values) and results of biochemical analyses (measured values) using Microsoft Excel; the Pearson's correlation coefficient for each correlation was calculated. Correlation with Pearson's correlation coefficients r lower than 0.5 was considered as poor, 0.5–0.7 as moderate, and 0.7–0.9 as good and more than 0.9 as an excellent.

Results

Fluorescence excitation ratio analysis

To assess the sensitivity of the FER method in diagnosing the changes/differences in the content of flavonoids in lettuce, the set of lettuce genotypes were exposed to two different environments and regularly monitored by the non-invasive method during two months (Fig. 1). The parameter expected to be sensitive to the accumulation of UV-absorbing compounds ($logFER_{R/UV}$, i.e., adjusted *FLAV* index) was increasing in both variants as a response to the increase of light intensities compared to initial cultivation in moderate light in a growth chamber (Fig. 1a–h). However, an increase of $logFER_{R/UV}$ was much higher in plants exposed to direct sunlight and more fluctuating temperature outside. The variability among genotypes was lower compared to differences caused by different environments; anyway, we observed some differences in genotypes both in the dynamics of $logFER_{R/UV}$ increase and in the final level of $logFER_{R/UV}$ before the harvest. There was no significant difference between green and red genotypes; interestingly, one of the green genotypes had a slower increase of $logFER_{R/UV}$, whereas the second one had relatively high $logFER_{R/UV}$ values. Very different trends were observed in the parameter $logFER_{R/G}$, corresponding to the content of green light absorbing compounds, mostly anthocyanins (Fig. 1i–p). As expected, a very high genotypic variability corresponded to the color form of the lettuce; the red genotypes had significantly higher values of $logFER_{R/G}$ compared to the green. The values of $logFER_{R/G}$ were not increasing significantly in green lettuce genotypes cultivated in greenhouse conditions. On the other hand, we observed some increase of $logFER_{R/G}$ in green plants exposed to direct sunlight. Anyway, the increase of $logFER_{R/G}$ was much higher in red genotypes, especially in variant grown outside. The trend and relative extent of the increase observed in $logFER_{R/G}$ in red genotypes was almost identical to $logFER_{R/UV}$ in red genotypes; it was not true for the green genotypes. The observed trends are in line with expectations.

The biochemical analyses and correlations with fluorescence parameters

Comparing the values of total phenolics and total flavonoids assessed by the standard biochemical assays with the values of two FER parameters derived from multispectrally induced chlorophyll fluorescence records on the day of harvest, app. 50 days after exposition at a given environment (Fig. 2) indicated that the general trends observed by the non-invasive methods are obvious also in the values of the biochemical parameters. In particular, in all genotypes, the exposition of plants to direct sunlight under fluctuating environmental conditions led to significantly higher content of total phenolics and flavonoids in lettuce. Moreover, we observed significant variability among the genotypes in content of total phenolics and flavonoid. The variability was partly associated with the color of the genotypes, where the red genotypes had a significantly higher content of total phenolics (in both variants) and flavonoids (in plants exposed to direct sunlight). Obviously, some of the trends observed in biochemical parameters do not fully correspond to the trends observed using non-invasive fluorescence technique, especially when assessing the red genotypes and $logFER_{R/UV}$ parameter.

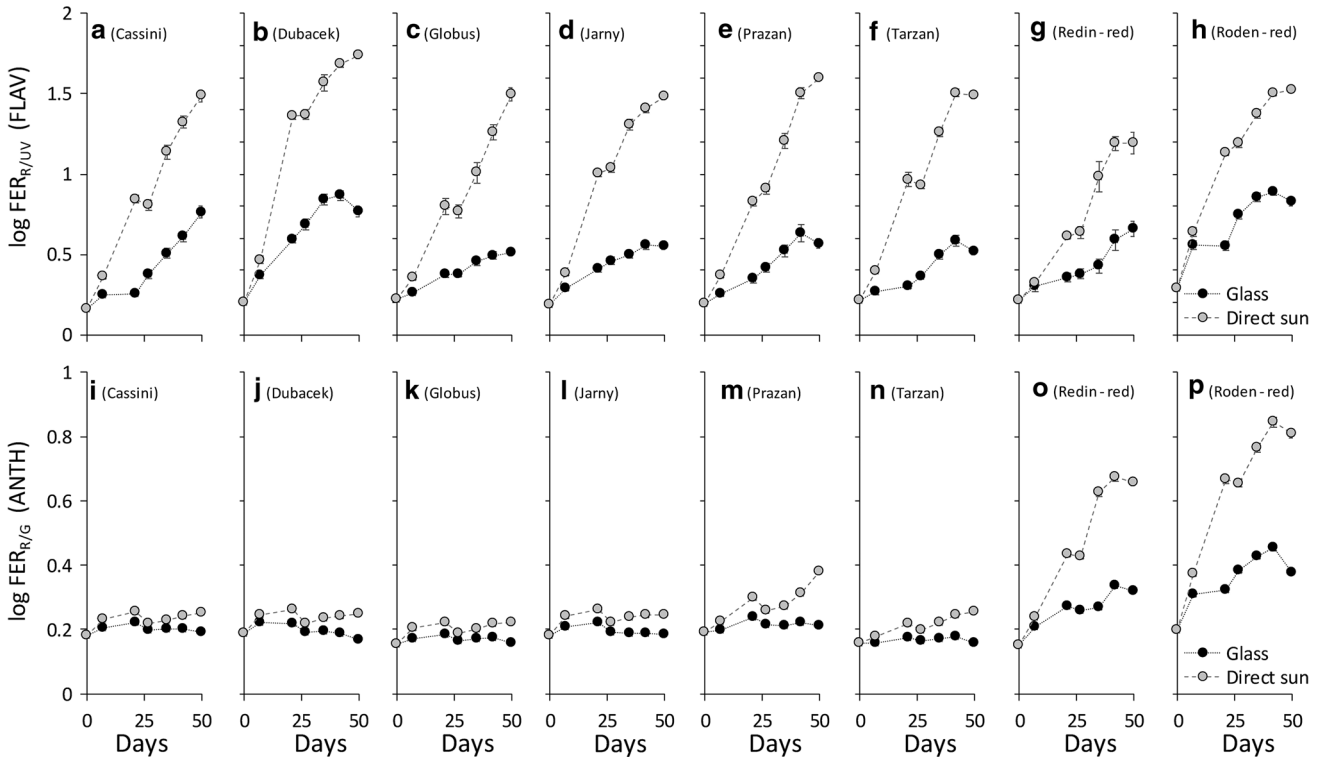

Fig. 1 The values of red/UV-induced fluorescence index—logFER$_{R/UV}$ (**a**–**h**) and red/green induced fluorescence index—logFER$_{R/G}$ (**i**–**p**) calculated from the records of multispectrally induced chlorophyll fluorescence in eight lettuce genotypes (six green and two red). At the beginning, plants were cultivated in a growth chamber under artificial light and in the stage of three leaves (day 0), a half of plants was exposed to direct sunlight and natural weather condition in a vegetation cage (variant "Direct sun") and the second group was placed inside behind the clear glass (greenhouse conditions) with a similar light intensity compared to the exterior (variant "Glass"). The measurements were done in 7–10 days interval. The measurements represent an average values ± SE from the records performed on all individual plants (each data point represents ~20 measurements)

To verify the relationships between the biochemical and the fluorescence parameters, we plotted the correlations of all values, regardless of the environment and genotype (Fig. 3). Obviously, we observed the positive correlations. The level of correlation can be classified as relatively good and significant, however, far away from an excellent correlation. Although the correlation between total flavonoids and anthocyanin index (Fig. 3d) has no direct relevance (because the flavonoid analysis covers the anthocyanins only marginally), the positive correlation indicates that the trends observed in flavonoids are similar also in other groups of polyphenols.

Discussion

Environmental and developmental effects on accumulation of phenolics in leaf epidermis

In natural conditions, phenolic compounds in epidermis serve as a screen protecting the leaf tissues from UV damage (Tevini et al. 1991; Jordan 2002). It is well known that there is almost no detectable solar UV-B radiation in the greenhouse due to its absorption by greenhouse glass (Teramura and Murali 1986). In the absence of UV radiation, the key enzyme of flavonoid synthesis, Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), has a low activity, leading to a significantly reduced synthesis of flavonoids in plant leaves (Krizek et al. 1998). In accordance with the results of different experiments (Kolb et al. 2001; Kolb and Pfündel 2005; Bidel et al. 2007; Morales et al. 2010, 2011; Latouche et al. 2013), the transfer of plants grown in low UV conditions into full sunlight induced an increase in epidermal UV-absorbing compounds, which is clearly evident on the kinetics of the logFER$_{R/UV}$ parameter. It was found that this accumulation of flavonoid compounds was specifically induced by UV-B light (Kolb et al. 2001; Bidel et al. 2007), which is efficiently filtered by common glass used in greenhouses. However, a significant (even much lower) increase of logFER$_{R/UV}$ was observed also after transferring the lettuce plants from a growth chamber into a greenhouse conditions, which confirms the observation of Agati et al. (2011) that also the intensity of visible light spectra determines the

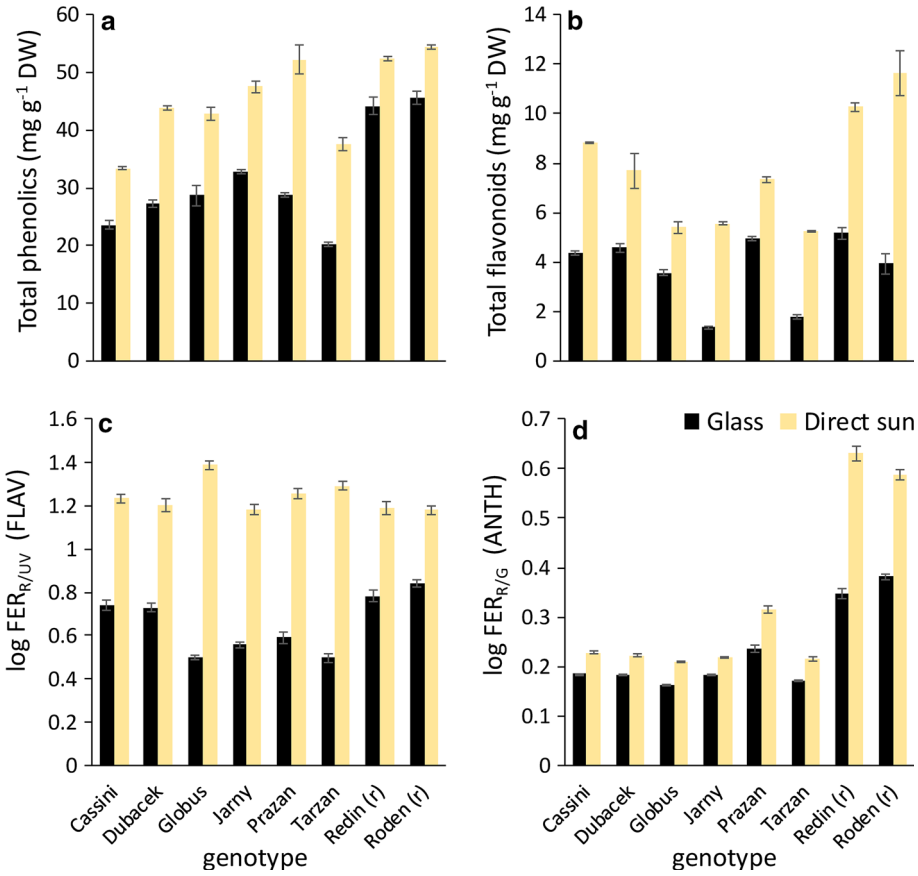

Fig. 2 The content of total phenolics (**a**), total flavonoids (**b**), values of red/UV-induced fluorescence index—log$FER_{R/UV}$ (**c**) and red/green induced fluorescence index—log$FER_{R/G}$ (**d**) in eight lettuce genotypes (six green and two red) assessed app. 50 days after the plants were exposed to two different environments: direct sunlight conditions in the vegetation cage (variant "Direct sun"), and greenhouse conditions, behind the clear glass, with a similar light intensity compared to the exterior (variant "Glass"). Mean values ± standard errors are presented. The values of fluorescence-based parameters were calculated from the records performed in a limited number of lettuce plants selected for biochemical analyses

accumulation of *FLAV* expressed in the values log$FER_{R/UV}$ parameter.

Whereas the kinetics of log$FER_{R/UV}$ recorded after exposure to direct sunlight was previously demonstrated by numerous authors (see previous paragraph), the kinetics of anthocyanin-related signal (parameter log-$FER_{R/G}$) in leaves was much less examined. To analyze efficiently the observations made in lettuce exposed to different environments, we need to focus separately on green and red genotypes. Interestingly, plants of green genotypes transferred to the greenhouse environment showed almost negligible changes in values of Log$FER_{R/G}$ parameter, despite the much higher light intensities. On the other hand, leaves of green genotypes exposed to direct sunlight had significantly higher Log$FER_{R/G}$ signal compared greenhouse conditions or initial value. It indicates some (even minor) synthesis of anthocyanins outdoors, which is, however, not induced significantly by the light intensity per se. Our results are in accordance with an observation that exposure to UV light promotes the production of foliar anthocyanin (Lindoo and Caldwell 1978).

In addition to generally expected increase of content of UV-absorbing compounds in leaves, the interesting is also the dynamics of the increase of log$FER_{R/UV}$ and log$FER_{R/G}$ signal during the experiment. To analyze the changes efficiently, we have calculated the daily rates of log$FER_{R/UV}$ and log$FER_{R/G}$ increase, which can serve as a measure of flavonoid/anthocyanin accumulation in leaf epidermis (Fig. 4). The kinetics of the flavonoid accumulation after transfer of plants from UV-free environment to direct sunlight typically exhibits a fast, exponential growth followed by the saturation period with slow growth only. We have observed a similar trend; however, the previous observation showed the period of steep flavonoid increase during the first few (4–5) days, following by a slow increase in the next period (Latouche et al. 2013). In our experiment, we have observed high rates of log$FER_{R/UV}$ increase even 40 days after the plants were transferred to direct sunlight. The same trend (but a lower rate) of the log$FER_{R/UV}$ increase was observed also in plants exposed to sunlight behind glass (indoor). There are two possible explanations of the sustained *Flav* increase: First, the plants were exposed to the gradually increasing intensity of solar radiation, as the experiment was performed under natural sunlight during a relatively long period of the spring season. The second possible reason of the long-lasting increase of the log$FER_{R/UV}$ signal can represent the developmental changes, as the experiments were starting with young plants having a very thin leaves and

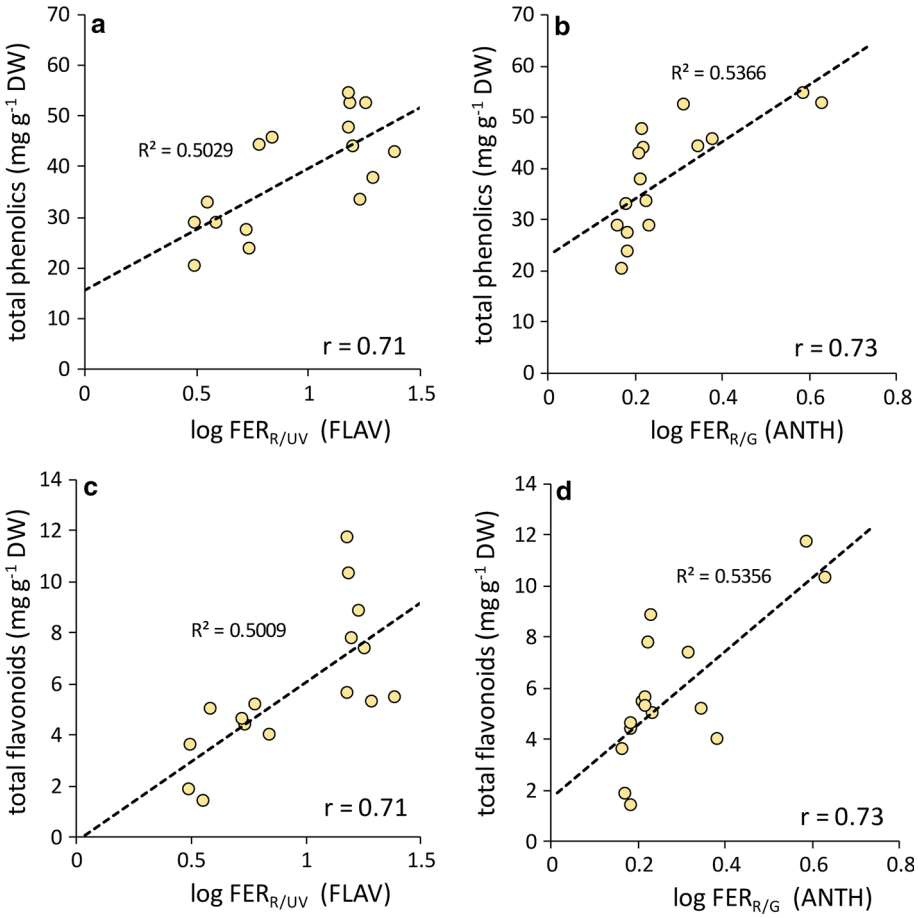

Fig. 3 Correlations between the indices of fluorescence excitation ratio analysis ($logFER_{R/UV}$, $logFER_{R/G}$) and the content of total phenolics (**a**, **b**), and total flavonoids (**c**, **d**). Each point represents a mean value of the parameters assessed eight lettuce genotypes grown in two different environments: direct sunlight conditions and behind the clear glass. The values *r* represents the Pearson's correlation coefficients for correlation

the developmental changes in leaf properties were evident. The effect of plant development on accumulation of phenolics in leaf epidermis was previously observed (Cerovic et al. 2002; Meyer et al. 2009) and its contribution to the increase of *Flav* signal is very probable. The trend of *Anth* signal was similar to Flav, but the duration of the high *Anth* accumulation (in red genotypes of lettuce) was shorter compared to Flav. In case of anthocyanins, it is interesting that in many species they occur in young leaves, whereas in later developmental stages the anthocyanin almost disappears (Karageorgou and Manetas 2006). This is not a case of lettuce, as the red forms of this vegetable are almost green in early growth phases and the anthocyanic phenotype occurs later.

Another important phenomenon, which is clearly visible on the kinetics of $logFER_{R/UV}$ and $logFER_{R/G}$ signal, is a temporary slowdown of *Flav* and *Anth* accumulation between 20th and 27th day of the experiment (Figs. 1, 4). This interval corresponds to a period, in which a significant increase of the temperature occurred; as in the first three weeks, the plants grown outdoors were exposed to temperatures below the optimum (Fig. 4d). In the temporary warmer period, the midday temperature was getting to the range which can be considered as the optimum for the growth of lettuce (20–24 °C). Despite the period was sunny and the nigh temperature was still quite low, both $logFER_{R/UV}$ and $logFER_{R/G}$ stopped to increase. It is well known that lower and fluctuating temperatures stimulate accumulation of phenolic compounds, especially anthocyanins (Lovdal et al. 2010). A fast accumulation of flavonoids and anthocyanins, which we observed mainly in early growth phases with suboptimal temperature, supports the hypothesis on the role of anthocyanins in protection against photoinhibition, which can be especially dangerous in low temperature conditions; therefore, the anthocyanins and flavonoids are important to prevent the negative effects of chilling stress (Havaux and Kloppstech 2001), especially associated with a general photoprotective function of anthocyanins in the photosynthetically active spectra (Mendez et al. 1999) as well as their radical scavenging activity (Gould et al. 2002).

In addition to a lower synthesis of secondary metabolites when the temperature is optimal, the enhanced growth rate may have diluting effect on the concentration of flavonoids. This is confirmed by the observations in green leaf genotypes (Fig. 4c), in which the values of $logFER_{R/G}$ indicated a decrease of concentration of

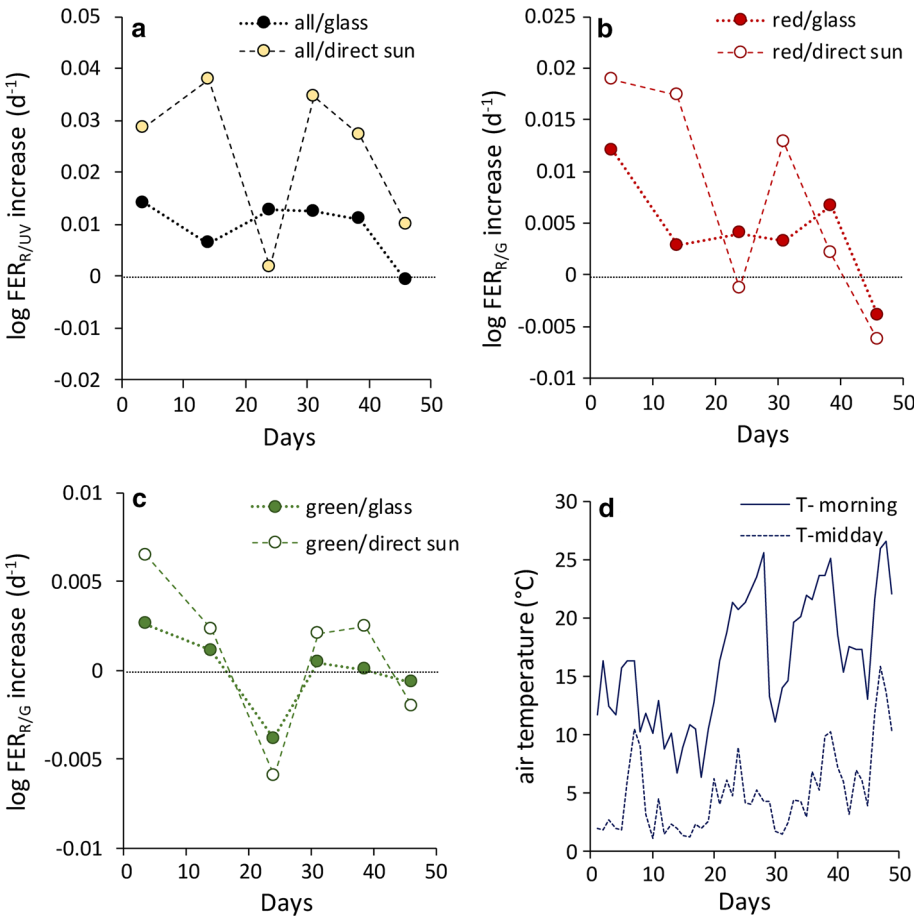

Fig. 4 Dynamics of signals related to the content of flavonoids (logFER$_{R/UV}$, **a**) or anthocyanins (logFER$_{R/G}$, **b**, **c**) expressed as an average daily increase of and for the individual intervals of measurements. The data for flavonoid index are presented as an average of all genotypes; the anthocyanin-related increase is presented separately for green genotypes (**b**) and red genotypes (**c**), based on the data presented in Fig. 1. Trend of mean morning, midday and average temperature for individual time intervals is presented

anthocyanins in the leaf epidermis. Interestingly, the same trend was observed also in the plants grown indoors, probably as an effect of temperature increase, too. Anyway, this trend was not observed in the same parameter in red leaf genotypes nor in the case of flavonoid accumulation in plants grown indoor (Fig. 4a, b).

The effects of temperature were confirmed by the subsequent increases of *Anth* and *Flav* accumulation rate after the weather become colder again. Analyzing the correlation between the air temperature character of the period prior to the measurements and the values indicating the rate of *Flav* and *Anth* accumulation (Table 1) showed the negative correlations, i.e., the increase in the temperature leads to the decrease in the rate of anthocyanin and flavonoid accumulation in lettuce. This relationship was highly significant in the case of anthocyanins in red leaf lettuce, in which the correlation coefficient reached value −0.95. On the other hand, lower level of correlations in other cases indicates that the temperature is not the only factor determining the content of phenolics; especially, the developmental stage and light intensity may strongly influence the accumulation of protective phenolic compounds in leaves.

Relevance of the fluorescence records for phenotyping aimed at flavonoid and phenolic content in lettuce

The cultivation of plants in greenhouse vs. field conditions is expected to produce different phenotypes in terms of flavonoid content, which was previously documented in numerous studies (Dumpert and Knacker 1985; Romani et al. 2002; Behn et al. 2010). The results of the biochemical analyses (Fig. 2) fully confirmed the expectations and were in line with the observations of other authors. For example, Tsormpatsidis et al. (2010) observed the decrease of flavonoids in lettuce due to low UV by more than 50%; it corresponds to our data. In general, we can state that the treatments and genotypes provided sufficient variability needed for testing the usefulness of the rapid non-invasive method for screening of genotypes in the breeding of lettuce, which was the main goal of our study.

One of the limiting steps in the process of crop improvement is the fact that the majority of the conventional methods is labor-intensive and costly. To solve this problem, the focus was turned on the phenomic approach, i.e. the development of high throughput, non-invasive novel technologies, methods and processes to provide

Table 1 The values of correlation coefficients between temperature characteristics and the parameters related to anthocyanin and flavonoid accumulation

Factor	$LogFER_{R/UV}$ increase (day^{-1})		$LogFER_{R/G}$ increase (day^{-1})	
	Green	Red	Green	Red
Mean temperature—morning	−0.47	−0.54	−0.48	−0.91*
Mean temperature—average	−0.63	−0.53	−0.67	−0.76
Mean temperature—midday	−0.74	−0.71	−0.69	−0.95*

* Statistically significant effects

information on important plant traits. The aim is to perform field evaluation of crops much faster, and facilitates a more dynamic, whole-of-lifecycle measurement less dependent on periodic destructive assays (Furbank and Tester 2011). The most promising are the techniques which can become a part of fully automated systems thanks to their non-invasive and non-contact applications. The fluorescence methods belong to the most promising tools in plant phenotyping and crop improvement (Harbinson et al. 2012; Brestic and Zivcak 2013; Kalaji et al. 2017) and the fluorescence excitation ratio (FER) method, though recently less utilized compared to other fluorescence applications, has met all requirements to be successfully applied in plant phenotyping (Sytar et al. 2016).

The unique property of the FER method is the ability to estimate the content of UV-absorbing compounds in the upper layers of the leaf (Bilger et al. 2001; Cerovic et al. 2002), which was clearly documented by the records obtained after plants were exposed to two different environments (Fig. 1). It is in accordance with previous observations showing the increase of UV-absorbing capacity due to an increase of UV radiation (Pfündel et al. 2007; Suthaparan et al. 2012; Müller et al. 2013). The value of the multispectrally induced chlorophyll fluorescence records is enhanced by the ability of the method to estimate the content of chlorophyll (Lejealle et al. 2010) and anthocyanins (Agati et al. 2007; Ghozlen et al. 2010). As the anthocyanins represent a fraction of the total phenolics and flavonoids, we assessed also the values of the index sensitive to their content, based on the absorption peak of anthocyanins in the green band of visible light (Figs. 1, 2). As expected, the red-colored lettuce genotypes had significantly higher values of $logFER_{R/G}$ compared to green genotypes, as well as the increase of anthocyanin content across the season, which corresponded with visual observations of enhancing the red color intensity.

Anyway, the presence of red genotypes caused the most evident discrepancies between the trends observed in biochemical analyses of phenolic compounds and total flavonoids and the values of FER parameters (Fig. 5a). In particular, we observed the highest content of total flavonoids and phenolics in red genotypes, which is in accordance with observations of Tsormpatsidis et al. (2010). This difference, however, was not expressed by the values of flavonoid index $logFER_{R/UV}$. The trends of correlations between $logFER_{R/UV}$ and total flavonoids were differing significantly between green and red genotypes. Obviously, the parameter $logFER_{R/UV}$ underestimated the content of total flavonoids in red varieties. This was caused by the fact that the measurements of UV-induced fluorescence (for calculation of the $logFER_{R/UV}$ index) were done with excitation at 375 nm, which is well absorbed by flavonols, but the anthocyanins had a low absorption in this range. On the other hand, anthocyanins contribute to absorption of UV radiation in the UV-B zone with (the absorption maximum ~280 nm (Qin et al. 2010) and they contribute to the total flavonoids and total phenolics assessed by the biochemical methods (Fig. 2a, b), although the contribution of anthocyanins to total flavonoids is non-specific (Mabry et al. 1970). Moreover, it was shown that the significant absorbance of anthocyanins at 630 nm (red LED emission) may lead to decrease of FRF_R/FRF_{UV} ratio, depending on the concentration of anthocyanins (Tuccio et al. 2011).

In this regard, the discrepancies between flavonoid/phenolics content and $logFER_{R/UV}$ values leading to lower correlation indices if the red genotypes are involved (Fig. 3) are expected and logical. To deal with this problem, we tried to find a solution how to improve the estimate to be useful for all genotypes. Our first attempt was aimed at eliminating the anthocyanin-related signal from the records of $logFER_{R/UV}$, adjusting the approach of Latouche et al. (2013), as it is described in "Materials and methods". A correlation plot of this corrected flavonoid index $FLAV_C$ with values of total flavonoids shows that the correction modified the values of $logFER_{R/UV}$ only marginally and the correlation was even lower compared to non-corrected parameter. It means that corrected *FLAV* index ($FLAV_C$) similarly to $logFER_{R/UV}$ index is, when the leaves are red, not so good estimate of flavonoid/phenolic contents, nor the content of UV-absorbing compounds.

As there is no other index published to solve this problem, we tried to modify the existing $logFER_{R/UV}$ index to add the effect of anthocyanins. We proposed the parameter Modified Flavonoid Index as:

$$MFI = \log[2FRF_R/(k_G \times FRF_G + k_{UV} \times FRF_{UV})],$$

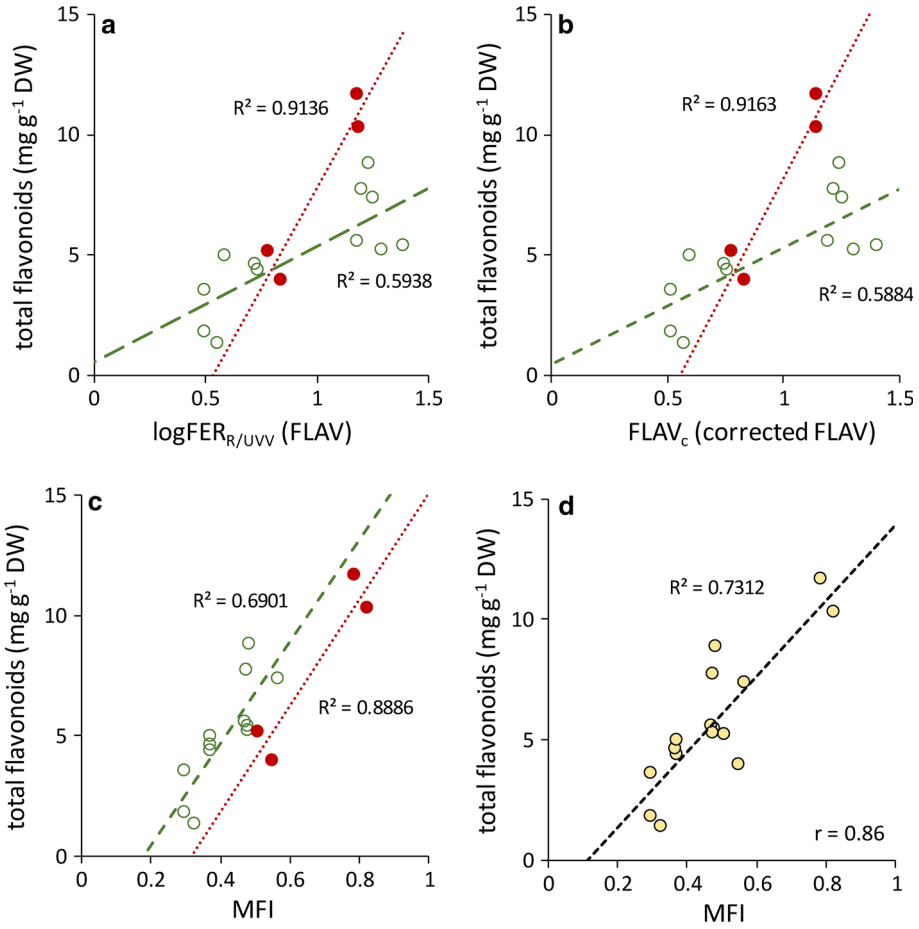

Fig. 5 The total flavonoid content found in observed genotypes plotted in relationship to **a** the *FLAV* index logFERR/UV, **b** corrected *FLAV* index $FLAV_C$, or **c**, **d** modified flavonoid index (MFI). In first three graphs (**a**–**c**), the correlations are displayed separately for green genotypes (*empty points*, *long-dashed trend line*) and red genotypes (*full points*, *dotted trend line*). Each point represents a mean value of the parameters assessed eight lettuce genotypes grown in two different environments: direct sunlight conditions and behind the clear glass. The value *r* represents the Pearson's correlation coefficients for correlation taking into an account all samples

where FRF_R, FRF_G and FRF_{UV} represent the far-red fluorescence signals measured after red (R), green (G) and UV excitation; determination of constants (k_G, k_{UV}) is explained in "Materials and methods".

In the log $logFER_{R/UV}$, the changes in flavonoid content are estimated by the ratio of the fluorescence signal measured after red to signal measured after UV excitation. In MFI, the UV-induced fluorescence signal is replaced by the average signal recorded after green and UV excitation, equal to $(FRF_G + FRF_{UV})/2$ (the number 2 then goes into the numerator in the final formula).

To demonstrate the physiological meaning of the correction of the flavonoid index, the formula for calculation of MFI can be rewritten as follows (omitting the constants k_G and k_{UV} for a better clarity):

$$\mathrm{MFI} = \log \mathrm{FER}_{R/UV} + \log\left[2 \times (\mathrm{FRF}_{UV})/(\mathrm{FRF}_{UV} + \mathrm{FRF}_G)\right],$$

As the $\log(1/x) = -\log(x)$, the MFI formula can be rewritten as:

$$\mathrm{MFI} = \log \mathrm{FER}_{R/UV} - \log\left[(1 + \mathrm{FRF}_G/\mathrm{FRF}_{UV}) \times 1/2\right].$$

The ratio FRF_G/FRF_{UV} can be multiplied by FRF_R/FRF_R, resulting in $(FRF_R/FRF_{UV})/(FRF_R/FRF_G)$, which in fact represents the ratio of two basic FER indices: $FER_{R/UV}/FER_{R/G}$. Thus, The MFI can be expressed as:

$$\mathrm{MFI} = \log \mathrm{FER}_{R/UV} - \log\left[\left(1 + \mathrm{FER}_{R/UV}/\mathrm{FER}_{R/G}\right) \times 1/2\right],$$

$$\mathrm{MFI} = \log \mathrm{FER}_{R/UV} - \log\left[\left(\mathrm{FER}_{R/G} + \mathrm{FER}_{R/UV}\right)/\mathrm{FER}_{R/G}\right) \times 1/2],$$

and, finally,

$$\mathrm{MFI} = \log \mathrm{FER}_{R/UV} + \log\left[2 \times \mathrm{FER}_{R/G}/\left(\mathrm{FER}_{R/G} + \mathrm{FER}_{R/UV}\right)\right].$$

Thus, the MFI can be expressed as a basic parameter ($logFER_{R/UV}$) plus correction, which is clearly expressed. As $FER_{R/UV}$ is proportional (even though non-linearly) to flavonoid content (*flav*) and $FER_{R/G}$ is proportional to anthocyanin content (*Anth*), it is obvious that the correction of basic $logFER_{R/UV}$ parameter represents a logarithmic function of the contribution of anthocyanins to the total pool of anthocyanins and flavonoids (i.e., *Anth*/*Anth* + *Flav*) measured simultaneously by the optical sensor. Theoretically, when the contribution of

anthocyanin is very high (red genotypes with a very low *Flav* values), the ratio [2 × $FER_{R/G}$/($FER_{R/G}$ + $FER_{R/UV}$)] can be close to 2 and the maximum theoretical positive correction of $logFER_{R/UV}$ can be achieved (MFI = -$logFER_{R/UV}$ + 0.301). This is exactly the case, when the signal of the fluorescence sensor ($logFER_{R/UV}$) will indicate the flavonoid content close to zero, but biochemical analyses will indicate some non-negligible content of flavonoids due to anthocyanins in the sample. Logically, the values of MFI will better match the results of the wet analyses. The opposite situation can occur when green genotypes having low *Anth* signal have a high *Flav* content; in this case, the [2 × $FER_{R/G}$/($FER_{R/G}$ + $FER_{R/UV}$)] will be between 0 and 1 and the logarithm will represent a negative value. In our case, the *Flav* signal was significantly higher than *Anth* signal (Fig. 1); therefore, the MFI for all our samples was lower than the original $logFER_{R/UV}$ values. This may raise a question whether the results are not diluted by this correction. Anyway, despite the numerical decrease, the relative differences among genotypes remained and the significance of the results was not negatively affected.

As it is evident from the graph (Fig. 5c), the trend of correlation of MFI parameter with total flavonoids in red and green genotypes was similar and the correlation index for green genotypes as well as for all genotypes (Fig. 5d) was significantly higher compared to original $logFER_{R/UV}$ parameter (Fig. 3). A better reflection of flavonoid and phenolic content is evident in time trends of MFI in lettuce genotypes exposed to different environments (Fig. 6).

Obviously, the values of parameter MFI better reflect the higher content of flavonoids in red genotypes compared to green indicated by the biochemical measurements. Compared to parameter $logFER_{R/UV}$, the difference in MFI values (Fig. 6) between "direct sun" and "glass" variants is not so dramatic, which more corresponds to the results of biochemical analyses. Thus, the MFI parameter seems to be a better proxy of the total flavonoids in leaves than the conventional flavonoid index, especially when the genotypes of different color are analyzed. This is especially important in a crop, such as lettuce, in which a huge genetic diversity exists, with numerous color forms, in which the anthocyanin content may range in a broad scale. Therefore, to be useful for screening and phenotyping, the methods and parameters providing reliable estimates in all genotypes in field conditions are needed. Based on our results, the fluorescence excitation ratio method using the MFI parameter seems to be promising for the applications in the field.

On the other hand, despite the use of modified index, the correlation cannot be denoted as excellent, as r value 0.86 corresponds to r^2 value 0.73. In our case, however, it must be noted that the excellent correlation can be hardly expected, for several reasons. First, the measurements were done non-invasively from the top of the plant and the measured sample only partially corresponds to the sample analyzed biochemically. The second reason is that the lettuce is quite heterogeneous biological material, with a corrugated surface, thick veins and unequal leaves.

The fact, which probably enables to reach a good level of correlation is that UV-absorbing flavonoids—flavonols and dihydroflavonols are located in epidermal cells (Gould et al. 2002), and, specifically, for red leaf genotypes of lettuce, the anthocyanins are restricted exclusively to the epidermis, too (Pfündel et al. 2007). On the other hand, the epidermal localization of phenolics is connected with the fact that measurements by the FER method produce surface-based information and, hence, the better correlation with measurements related to leaf area units can be expected.

It must be taken into an account that also the biochemical methods generally used in field studies and in food chemistry are not fully precise. Total phenolic content obtained by Folin–Ciocalteu reagent and total flavonoid content obtained by the aluminum chloride method cannot fully cover the fact that each lettuce variety will react to sunlight by increasing each flavonol and anthocyanin compound in various proportions. To be fully precise, the full polyphenol profile and calculation of each component using their own absorption coefficient when complexed with Al_3^+ or with Folin–Ciocalteu reagent is needed. But this is very difficult and laborious approach, and interpretation of the results is not straightforward. Another possibility is to be focused on very precise estimates of some main phenolic components; this approach has, however, also the limited accuracy, as our optical sensor provides an information based on a mix of signals, representing a result of all the UV-absorbing (mostly flavonoid) and green absorbing (mostly anthocyanins) compounds. Therefore, we found as meaningful to be focused on the parameters which also provide some kind of integrated information instead of partial compounds, taking into an account some possible impreciseness.

Another factor limiting the correlations between measured fluorescence signals and the content of flavonoids/phenolics can be the interferences with other metabolites. For example, the presence of some red pigments (other than anthocyanins) can seriously influence the values of green excited fluorescence ratio ($logFER_{R/G}$). One of the possible candidates to cause interferences in lettuce is carotenoids; however, it was found that only carotenoids not involved in light harvesting (out of the chloroplast) influence the fluorescence ratios (Pfündel et al. 2007). This can be problematic in some fruits (fruit peels), where the non-chloroplastic carotenoid to chlorophyll ratio can be very high. However, we do not expect the serious impreciseness in estimates of flavonoids and phenolics due to

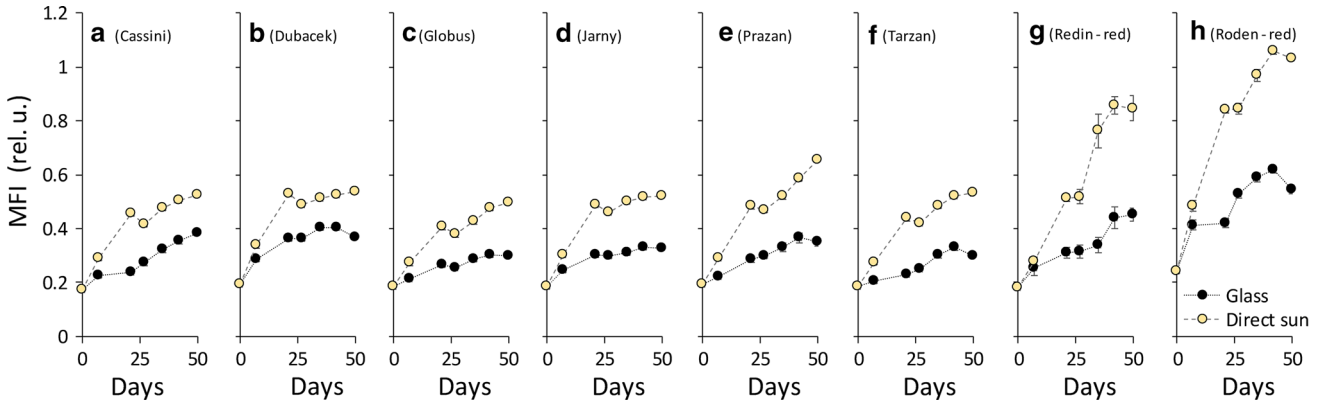

Fig. 6 The values of the modified flavonoid index—MFI calculated from the records of multispectrally induced chlorophyll fluorescence in eight lettuce genotypes (six green and two red). At the beginning, plants were cultivated in a growth chamber under artificial light and in the stage of three leaves (day 0), a half of plants was exposed to direct sunlight and natural weather condition in a vegetation cage (variant "Direct sun") and the second group was placed inside behind the clear glass (greenhouse conditions) with a similar light intensity compared to the exterior (variant "Glass"). The measurements were done in 7–10 days interval, in all plants grown within the experiment (using the same dataset as in Fig. 1)

genotypic differences in carotenoid content in lettuce. Nevertheless, the extent of the effects of interferences with other metabolites on the preciseness of the estimate should be assessed experimentally in detailed research in individual crops.

Considering these factors, the achieved level of correlation can be regarded as relatively good and promising. Although the method has not ambition to detect subtle differences between the similar genotypes grown in the same conditions, it can be useful to identify the candidates, which can be subjected to the more precise analyses in the next step. For its rapidity and simplicity, the method can be well applied in QTL studies aimed at phenolic compounds (Kaushik et al. 2015), as well as in genotype × environment × management studies (Asseng and Turner 2007), where the enormous number of measurements needs to be done efficiently. Moreover, in addition to the field selection, the method can be potentially utilized also for alternative applications, such as determination optimum harvesting time or following the changes during processing and storage. As the method is non-invasive and non-contact, it can be used either as a hand-held device or easily adjusted to become a part of the fully automated system. Therefore, in addition to field use, the future works can also be aimed at testing the changes in postharvest or processing of leafy vegetables. Thus, the sensor can become a part of fully automated laboratory or field phenotyping systems, contributing significantly to the crop improvement, storage and processing of leafy vegetables and other crops.

Author contribution statement MZ KB, and OS wrote the paper. MZ conducted the statistical analysis. KB and OS conducted biochemical analyses and fluorescence measurements. MB, KO and SIA contributed to experimental design and interpretation of results, and helped to draft the manuscript. Neither the manuscript nor any part of its content has been published or submitted for publication elsewhere. All authors read and approved the final manuscript.

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