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Photochemistry of thylakoid membranes in two pea cultivars with different leaf colouration

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Abstract Photochemical features of thylakoid membranes and chlorophyll content were investigated in two pea (*Pisum sativum* L.) cultivars with different leaf colouration: 'Assas' (red leaves) and 'Arvika' (green leaves). In vivo measured chlorophyll fluorescence (OJIP) was used to evaluate overall photosynthetic efficiency (expressed as performance index, PI_{ABS}) as well as for advanced analysis of main photochemical processes per active reaction centre (RC) of photosystem II (PSII). To evaluate the response of PSII driven linear electron transport (relETR) and non-photochemical quenching (NPQ), the plants were challenged with short-term high light ($\sim$2000 μmol m^{-2} s^{-1}). In spite of prevailing red colour, leaves of 'Assas' had identical concentrations of chlorophylls *a* and *b* as green ones. The OJIP transients showed that red leaves grown in field (500–800 μmol m^{-2} s^{-1}) had decreased PI_{ABS} and electron transport per RC beyond primary acceptor (Q_A^-) compared to green leaves. After high light exposure red leaves revealed impaired relETR accompanied with increased NPQ values. Anthocyanins located in epidermal cells affected neither chlorophylls concentrations nor the light capture features of PSII. Despite equal concentrations of chlorophylls and PSII photochemistry further than Q_A^- in both leaf types, red leaves reduced overall photosynthetic efficiency due to impaired relETR in thylakoid membranes.

Keywords *Pisum sativum* L. · Anthocyanins · Chlorophyll fluorescence · Photosynthesis · Photoprotection

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1 Introduction

Anthocyanins are the group of water soluble plant pigments responsible for red, purple and blue colour during various periods of plant development, such as juvenile or senescing phases, as well as for response to various environmental disturbances like low temperature, high light, drought or salinity (Cooney et al. 2015; Landi et al. 2015; Ranjan et al. 2014). Anthocyanin biosynthesis is stimulated by various signals from the environment (temperature, wounding, nutrient deficiency, etc.), but the most significant role in biosynthesis of anthocyanins is attributed to light (Das et al. 2011). Visible and UV-light are responsible for accumulation of the specific anthocyanin compounds that absorb fractions of blue, green and UV wavelengths and, in that way, contribute to the protection from excessive irradiation, and reduce damage to photosystem II (PSII) (Gould 2004; Landi et al. 2015).

Red colour of the most vegetative and reproductive plant organs originates from anthocyanins (Manetas 2006). In leaves, they accumulate in vacuoles of adaxial (upper) epidermis and mesophyll cell layers and, less often, in abaxial (lower) epidermis (Hughes et al. 2014; Landi et al. 2015; Liakopoulos et al. 2006). Accumulation of anthocyanins in adaxial epidermis is of great importance in ecological defence and photoprotection. Ecological defence implies that anthocyanins reduce damage caused by potential herbivores or pathogens (Gould 2004; Hughes and Lev-Yadun 2015). Additionally, the role of anthocyanins is photoprotection of photosynthetic tissues susceptible to excessive light. Red leaves serve as filter that absorbs green light thus diminishing the damage to photosystems because such filtered light that comes to the chloroplasts produce less reactive oxygen species (Gould 2004; Tattini et al. 2014). It was suggested that photosynthetic cells provide signalling molecules that are regulated by plastoquinone pool redox state. In that way, high light conditions may indeed elicit the accumulation of anthocyanins in leaves what provides efficient protection for photosynthetic apparatus against excessive light and, in that way, mitigate photo inhibition (Das et al. 2011). Beneficial role of abaxial anthocyanin accumulation was recently suggested by Hughes et al. (2014). They proposed that abaxial anthocyanins participate in leaves photoprotection without reducing the photosynthetic capacity, which is advantageous in shade conditions with the occasional appearance of high light sunflecks.

Although physiological role of anthocyanins is the subject of numerous recent investigations (Dewez and Perreault 2013; Landi et al. 2015; Solovchenko and Chivkunova 2011; Tanino et al. 2014; Tsormpatsidis et al. 2010), their exact role in regulation of photosynthetic processes remain to be elucidated. Photosynthetic processes are the most sensitive to changes in environmental conditions. Energy absorption and redistribution between two photosystems needs to be highly balanced in order to function properly. Under low or moderate light, it is assumed that the balance between absorption and utilization of energy is achieved. In cases when moderate light is combined with some other disturbances such as low temperature or high light, it can reduce the utilisation of absorbed light energy (Steyn et al. 2009). Increased accumulation of anthocyanins in both, juvenile and mature leaves under excessive light could efficiently reduce the risk of photoinhibition (Fondom et al. 2009; Landi et al. 2015). Solovchenko and Chivkunova (2011) recently showed positive correlation between the electron transport rate and anthocyanins content at lower light intensities in common hazel juvenile red leaves. They also suggested that anthocyanins protect photosynthetic apparatus from oxidative damage by reducing the absorption of excessive light that would be normally absorbed by chlorophylls and carotenoids. On the other hand, Peng et al. (2006) reported limited electron transport rate and increased non-photochemical quenching (NPQ) in purple rice leaves suggesting increased photoprotection in photooxidative conditions. Photoprotective influence on PSII was reported by Dewez and Perreault (2013) supporting the role of anthocyanins in regulation of PSII energy dissipation processes in red *Tradescantia* leaves. Our previous investigation on the photosynthetic apparatus functioning of red and green leaves from "Crimson King" Norway maple tree (Štolfa et al. 2006) revealed fully functional PSII in both leaf types although red leaves had lower O_2 production. Further, lower Chl *a* to Chl *b* ratio and increased number of thylakoids per granum in red leaves suggested that they acted as shade ones.

Pea (*Pisum sativum* L.) cultivars with different leaf colouration, cv. 'Assas' and cv. 'Arvika', show good tolerance to low temperatures and early frost. Spring pea cultivar 'Arvika' has green leaves while winter cv. 'Assas' have distinctive red leaves colouration in

foliar stage of development. Red colouration of leaves disappears with maturity of the plant. Our preliminary investigations confirmed differences in leaf colour using the leaf sections where the presence of anthocyanins were detected in red leaves. Histological localisation of anthocyanins in a number of studies was accompanied by determination of their content showing that anthocyanins content to be dominant over chlorophylls (Landi et al. 2014; Merzlyak et al. 2008; Peng et al. 2006).

Considering the leaf colour of two preliminary investigated pea cultivars, our aim was to search differences in primary photosynthesis events connected with visible presence of anthocyanins. Thus, the chlorophylls concentrations, overall photosynthetic activity, photochemical and non-photochemical processes per active reaction centre of PSII (ABS, TR_0, DI_0 and ET_0) were determined. In order to avoid possible photoinhibition, measurements were performed at moderate light intensity because moderate light intensity provides optimal conditions for photosynthesis performance (Dewez and Perreault 2013; Landi et al. 2014; Osorio et al. 2013; Steyn et al. 2009). However, photosynthetic processes could be somewhat limited due to the fact that anthocyanins can attenuate photosynthetically active radiation. Although it is unfavourable in normal conditions, this feature of red leaves might be beneficial under stressful high light conditions (Gould et al. 2010). In order to evaluate green and red pea leaves in response to high light, the PSII driven electron transport rate (relETR) and non-photochemical quenching (NPQ) were investigated.

Here we hypothesized two possible scenarios of photosynthetic regulatory mechanisms that might appear as the consequence of anthocyanins accumulation in red leaves in comparison to the green ones: (1) it might affect chlorophyll accumulation and consequently, photochemistry of PSII due to changes in light absorption processes; and (2) it might reduce photosynthetic efficiency at high light via reducing thylakoid electron transport.

2 Materials and methods

2.1 Plant material and growth conditions

Two pea (*Pisum sativum* L.) cultivars, cv. 'Assas' and cv. 'Arvika', were grown on euteric cambisol soil in an experimental field (45°34′N, 18°42′E). Plants were investigated at foliar developmental stage (3–4 leaves) during March 2012, when cv. 'Assas' still produced red leaves. Fully developed leaves (third real leaf from the top) of both cultivars were sampled between 7:00 and 8:00 h under natural irradiance (500–800 μmol m^{-2} s^{-1}) with day/night cycles of about 8/16 h. Five cm above the ground, average temperature was 1.6 °C. Fast chlorophyll *a* fluorescence was measured in the field, whereas for other analyses plants were collected, transported to the laboratory and immediately used for analyses.

2.2 Experimental strategy

Due to the visible difference in leaf colouration between cultivars 'Arvika' and 'Assas', preliminary investigations using hand cut leaf sections were performed to confirm the presence of anthocyanins. After localization of anthocyanins, the fast chlorophyll *a* fluorescence was measured in the field at moderate light intensity to avoid photoinhibition. The same leaves were used for chlorophyll *a* fluorescence measurements, determination of photosynthetic pigments concentration and analysis of acetone pigment extracts absorption spectral characteristics. In order to acquire detailed insight into functioning of photosynthetic apparatus in two pea cultivars, we analysed chlorophyll *a* transients and OJIP test parameters. Further, same category of leaves were collected in the field and treated with short-term high irradiation using saturation pulse method in laboratory to estimate the response of photosynthetic apparatus of two pea cultivars at high light conditions.

2.3 Anthocyanin localization

The middle parts of a fresh leaves were hand cut with razor blade and transverse leaf sections were placed in distilled water. The localization of anthocyanins was observed using light microscope (Carl Zeiss, Jena, Germany) and photographed with digital camera (Olympus FE-115, China).

2.4 Photosynthetic pigments determination and characteristic of absorption spectra

Both leaf types were sampled to analyse photosynthetic pigments. Six randomly selected leaves from

different plants were combined in a composite sample and six replicates were taken for each analysis. After removal of main veins, leaf tissue was macerated into fine powder with liquid nitrogen. Powdered plant material was extracted with the cold absolute acetone at 4 °C and then re-extracted several times until it was completely uncoloured. Acetone extracts from both, red and green leaves were scanned at 330–900 nm by UV–Vis spectrophotometer (Specord 40, Analytik Jena, Germany) to obtain the total pigment spectra. The same acetone extracts were used to determine the concentrations of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) spectrophotometrically at 661.6 and 644.8 nm. The chlorophylls *a* and *b* ratio (Chl *a/b*) and total chlorophylls (Chl $a + b$) concentration were calculated according to Licthenthaler (1987).

2.5 Fast chlorophyll *a* fluorescence

Photosynthetic activity was measured on ten randomly selected leaves of each cultivar using Plant efficiency analyzer (PEA, Hansatech, UK). All measurements were performed on fully dark-adapted leaves in the field using lightweight leaf clips with shutter. Immediately after 30 min of dark adaptation, the leaves were exposed to a pulse of saturating red light (3200 μmol m^{-2} s^{-1}, peak at 650 nm). OJIP-test was used to calculate biophysical parameters that quantify stepwise energy flow through PSII (Strasser et al. 2000; Strasser et al. 2004). Therefore, the following recorded data were used: F_0 (fluorescence intensity at 50 μs), F_m (maximal fluorescence intensity), F_{300} (fluorescence intensity at 300 μs), F_I (fluorescence intensity at 2 μs) and F_J (fluorescence intensity at 30 μs) to calculate quantum yield of primary photochemistry (TR_0/ABS or F_v/F_m), density of reaction centers (RC) (Q_A^- reducing RC of PSII; RC/CS_0), variable fluorescence at J and I step (V_J, V_I), energy distribution through PSII per RC (ABS/RC, TR_0/RC, ET_0/RC and DI_0/RC) as well as performance index (PI_{ABS}) together with three related parameters (RC/ABS, TR_0/DI_0 and $ET_0/(TR_0-ET_0)$) (Strasser et al. 2000) (Table 1). Further, in order to convert data into a form that they can be compared, double normalization between O and P steps was used. The O-P normalization gives us relative variable fluorescence $W_{OP} = (F_t - F_0)/(F_m - F_0)$ which measures the fraction of reduced Q_A at any time (t). Fluorescence data were plotted on logarithmic time scale (from 50 μs to 1 s) and O, J, I and P steps were marked in plots.

2.6 Saturating pulse method

The effect of light intensity on PSII activity was determined by measuring chlorophyll *a* fluorescence in vivo using amplitude modulated fluorometer (Mini-PAM, Walz, Germany). Pea leaves were taken in the field, immediately transferred in laboratory in portable fridge at +4 °C, dark-adapted for 30 min and measured. Minimal (F_0) and maximal (F_m) fluorescence yields were measured using dark-adapted leaves. The F' and F_m' parameters were measured at the photosynthetically active photon flux density (PPFD) of 100, 500, 1200 and 2000 μmol m^{-2} s^{-1} serving as short-term treatments. Effective quantum yield of PSII ($\Delta F/F_m'$), relative linear electron transport (relETR) and non-photochemical quenching (NPQ) were calculated according to Schreiber et al. (1994).

2.7 Data analysis

Statistical differences between green and red leaves were analysed using Student's *t* test modified for small samples. Asterisk (*) indicates significant difference between compared parameters in red and green leaves. Results were expressed as arithmetic means of 6 replicates ± standard error of means (SE) except for measurement of chlorophyll *a* fluorescence when 10 replicates were used. Differences were considered significant at $P < 0.05$. For all statistical analyses Statistica 8.0 software (StatSoft, Inc. 2007) was used.

3 Results

3.1 Anthocyanin localization in leaves

Leaf morphology of the two pea cultivars differed mainly in leaf colouration. Cultivar 'Arvika' (Fig. 1a) showed green leaves without any sign of red colouration while leaves of cv. 'Assas' (Fig. 1b) showed apparent red colouration. Transverse sections of pea cv. 'Arvika' (Fig. 1c) showed no anthocyanins in vacuoles while cv. 'Assas' (Fig. 1d) revealed that anthocyanins were mainly located in the vacuole of upper (adaxial) and lower (abaxial) epidermis.

Table 1 OJIP test parameters and expressions

Recorded and technical parameters	Description
F_0	Minimal fluorescence intensity (50 μs)
F_m	Maximal fluorescence intensity—P step;
F_{300}	Fluorescence intensity at 300 μs
F_J	Fluorescence intensity at 2 ms—J step
F_I	Fluorescence intensity at 30 ms—I step
F_v	Maximal variable fluorescence; F_m–F_0
V_t	Relative variable fluorescence at time t; $(F_t-F_0)/(F_m-F_0)$
V_J	Relative variable fluorescence at time J step; $(F_J-F_0)/(F_m-F_0)$
V_I	Relative variable fluorescence at time I step; $(F_I-F_0)/(F_m-F_0)$
M_0	Approximated initial slope of relative variable fluorescence F_v; $(dV/dt)_0$
TR_0/ABS	Maximum quantum yield of PSII; $1-(F_0/F_m) = F_v/F_m$
RC/CS_0	Density of active RC per cross section;$F_v/F_m \times (V_J/M_0) \times F_0$
ABS/RC	Absorption flux per active RC; $M_0 \times (1/V_J) \times [1/(F_v/F_m)] = (TR_0/RC)/(TR_0/ABS)$
TR_0/RC	Trapping flux per active RC; $M_0 \times (1/V_J)$
ET_0/RC	Electron transport flux per active RC; $M_0 \times (1/V_J) \times (1-V_J) = (TR_0/RC) \times (ET_0/TR_0)$
DI_0/RC	Dissipation flux per active RC; $(ABS/RC) - (TR_0/RC)$
PI_{ABS}	Performance index on absorption basis; $(RC/ABS) \times (TR_0/DI_0) \times [ET_0/(TR_0 - ET_0)]$
RC/ABS	Density of RC on chlorophyll *a* basis; $(RC/TR_0) \times (TR_0/ABS) = \left[(F_J - F_0)/4\left(F_{300\mu s} - F_0\right)\right] \times (F_v/F_m)$
TR_0/DI_0	Flux ration trapping per dissipation; F_v/F_0
$ET_0/(TR_0\text{-}ET_0)$	Electron transport beyond Q_A^-; $(F_m - F_J)/(F_J - F_0)$

3.2 Photosynthetic pigment concentrations and absorption spectra

Chlorophylls, Chl *a* ($t = 1.337$, $P = 0.211$), Chl *b* ($t = 1.466$, $P = 0.173$), Chl *a* + *b* ($t = 1.454$, $P = 0.177$) concentration as well as Chl *a*/*b* ratio ($t = 1.415$, $P = 0.187$, Table 2) showed no significant difference between green and red pea leaves. Although the absorption spectra of leaf extracts revealed some similarities (Fig. 2), there were also some marked differences between red and green leaves. These differences can be attributed to the presence of anthocyanins observed in leaf sections. The acetone extracts from green leaves showed higher peaks in blue (430 nm) and red (660 nm) part of the absorption spectra (scanned at 330–900 nm), while acetone extracts from red leaves revealed additional peak at 360 nm (UV–A region) and had slightly lower absorption in blue and red part of spectra.

3.3 Photosynthetic performance

Minimal, F_0 ($t = 0.815$, $P = 0.426$) and maximal, F_m ($t = 0.721$, $P = 0.480$) fluorescence intensity, as well as maximum quantum yield of PSII, F_v/F_m ($t = 2.044$, $P = 0.056$)) and density of active reaction centres per cross section, RC/CS_0 ($t = 1.111$, $P = 0.281$) showed no significant difference between green and red pea leaves (Table 3).

Both green and red pea leaves revealed characteristic shape of OJIP transients. Significantly higher variable fluorescence at J step, V_J ($t = 2.115$, $P = 0.048$) and I step, V_I ($t = 5.515$, $P < 0.05$) was observed in red pea leaves (0.450 for V_J and 0.724 for

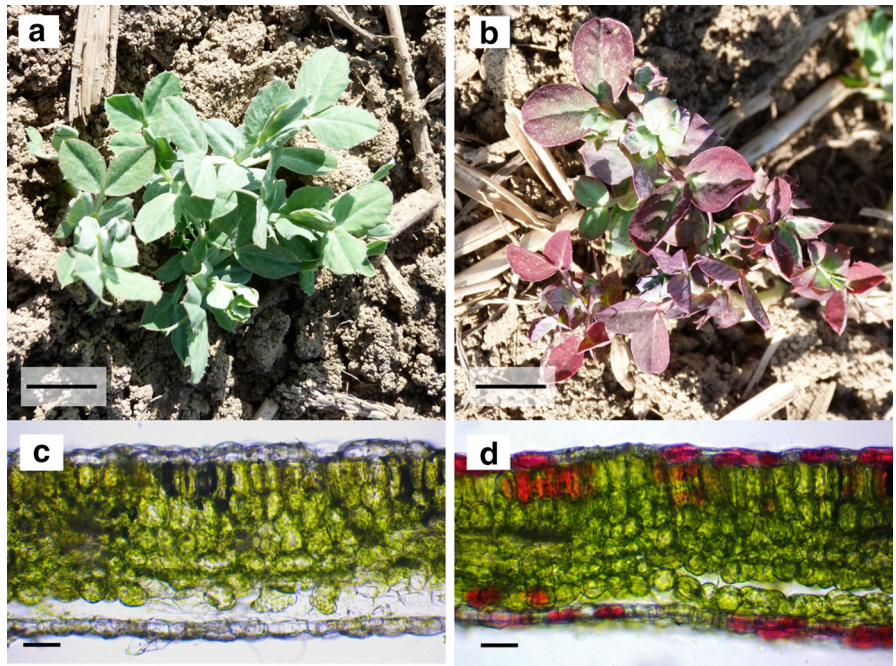

Fig. 1 Morphology and leaf anatomy of two pea (*Pisum sativum* L.) cultivars: 'Arvika' (**a**, **c**) and 'Assas' (**b**, **d**). Photographs of the intact pea plants were taken in the field before measurements (**a**, **b**); *bars* 2 cm. Anthocyanins localization in transverse sections of pea leaves (**c**, **d**); *bars* 100 μm

Table 2 Photosynthetic pigment content in green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves

	Chl *a* (mg/g^{-1} FW)	Chl *b* (mg/g^{-1} FW)	Chl *a* + *b* (mg/g^{-1} FW)	Chl *a/b*
cv. 'Arvika'	1.311 ± 0.064	0.687 ± 0.076	1.997 ± 0.135	1.977 ± 0.139
cv. 'Assas'	1.214 ± 0.034	0.553 ± 0.051	1.766 ± 0.085	2.261 ± 0.145
t-value	1.337	1.466	1.454	1.415
P-value	0.211 (n.s.)	0.173 (n.s.)	0.177 (n.s.)	0.187 (n.s.)

Data represents mean ± SE of six replicates, significant difference was considered at $P > 0.05$

Chl a chlorophyll a, *Chl b* chlorophyll *b*, *Chl a* + *b* total chlorophyll content, *Chl a/b* chlorophyll *a* to chlorophyll *b* ratio

n.s indicate no significant difference according to the Student's *t*-test

V_I) compared to green ones (0.421 for V_J and 0.676 for V_I, Fig. 3c).

Specific activities (fluxes) per active reaction centre measured in green and red pea leaves are shown in Fig. 4. Absorption, ABS/RC ($t = 0.286$, $P = 0.778$), trapping, TR_0/RC ($t = 0.873$, $P = 0.394$) and dissipation, DI_0/RC ($t = 1.343$, $P = 0.196$) showed no significant difference between green and red pea leaves. On the contrary, electron transport beyond Q_A^-, ET_0/RC ($t = 3.420$, $P = 0.003$) in red leaves (1.025) was significantly decreased in comparison to green pea leaves (1.100).

Decreased PI_{ABS} ($t = 2.752$, $P = 0.013$) values were observed in red (2.915) leaves compared to green ones (2.390) (Fig. 5). Decreased values of trapping to dissipation ratio, TR_0/DI_0 ($t = 2.248$, $P = 0.037$) and efficiency of excitation energy conversion to electron transport, $ET_0/(TR_0 - ET_0)$ ($t = 2.178$, $P = 0.043$) contributed to lower PI_{ABS} in red leaves because density of reaction centres on Chl basis, RC/ABS ($t = 0.251$, $P = 0.805$) in red leaves showed no significant difference compared to green pea leaves (Fig. 5).

Red pea leaves showed significantly lower PSII driven relETR values measured at all four PPFDs [100 ($t = 2.540$, $P = 0.029$), 500 ($t = 3.887$, $P = 0.003$), 1200 ($t = 2.368$, $P = 0.039$) and 2000 μmol m^{-2} s^{-1} ($t = 3.283$, $P = 0.009$)] compared to green leaves

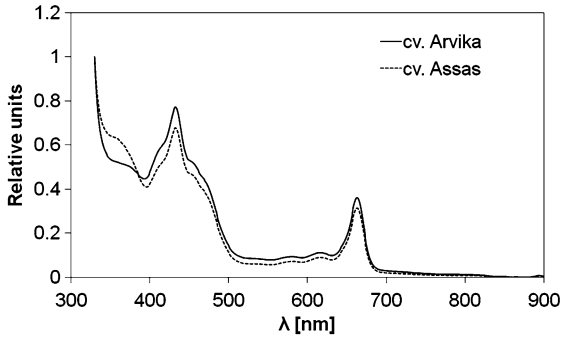

Fig. 2 Absorption spectra of total pigment acetone extracts from green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves. Absorption spectra were double normalized between 330 and 900 nm. Measurements were performed six times and the average is shown

(Fig. 6a). On the other hand, only the highest light intensity, 2000 μmol m^{-2} s^{-1} ($t = 4.771$, $P < 0.05$) triggered significantly higher non-photochemical quenching (NPQ) (Fig. 6b) in red pea leaves (2.088) when compared to green ones (1.193). Effective quantum yield of PSII ($\Delta F/F_m'$) was significantly lower in red pea leaves at 1200 ($t = 2.368$, $P = 0.039$) and 2000 μmol m^{-2} s^{-1} ($t = 3.283$, $P = 0.009$) compared to green ones. $\Delta F/F_m'$ measured at 1200 μmol m^{-2} s^{-1} was 0.569 and 0.486 in green and red reaves, respectively, while at 2000 μmol m^{-2} s^{-1} green leaves showed 0.478 and red leaves exhibited 0.358.

Table 3 Chlorophyll *a* fluorescence parameters in green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves

	F_0	F_m	F_v/F_m	RC/CS_0
cv. 'Assas'	343.017 ± 10.411	1983.454 ± 60.000	0.827 ± 0.002	148.922 ± 3.351
cv. 'Arvika'	355.608 ± 11.416	1930.920 ± 41.283	0.816 ± 0.005	155.767 ± 5.173
t value	0.815	0.721	2.044	1.111
P value	0.426 (n.s.)	0.480 (n.s.)	0.056 (n.s.)	0.281 (n.s.)

F_0 minimal fluorescence intensity; F_m maximal fluorescence intensity; F_v/F_m maximal quantum yield of PSII; RC/CS_0 density of reaction centres

Data represents mean ± SE of ten replicates, significant difference was considered at $P > 0.05$

n.s indicate no significant difference according to the Student's *t*-test

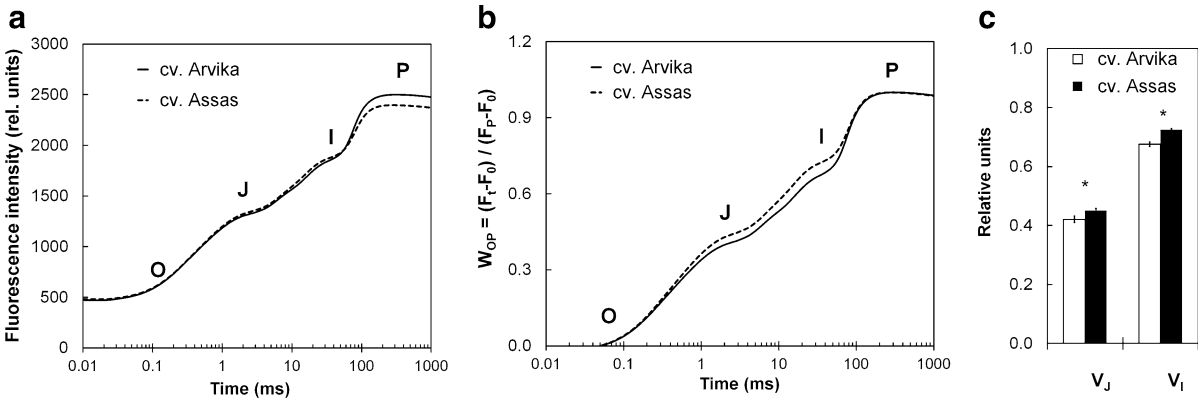

Fig. 3 Chlorophyll *a* fluorescence kinetics (OJIP) in dark adapted green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves. Each native curve (**a**) and double, O–P normalized transients ($W_{OP} = (F_t - F_0)/(F_P - F_0)$) (**b**) represents average kinetics of 10 replicates, plotted on logarithmic time scale. The O, J, I and P steps are marked in the plots. Relative variable fluorescence at J (V_J) and I (V_I) step (**c**) are expressed in relative units, columns represent mean values and bars are SE of 10 replicates. Significant difference was considered at $P < 0.05$ according to the Student's *t*-test; *asterisk* denotes significant difference

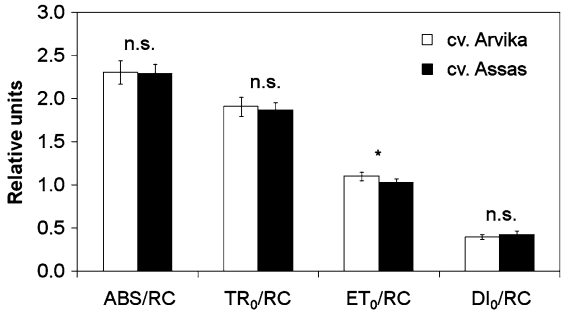

Fig. 4 Specific fluxes or specific activities per active reaction centre measured in green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves. All parameters are expressed in relative units. Columns are mean values and bars are SE of 10 replicates, significant difference was considered at $P < 0.05$ according to the Student's *t*-test; *asterisk* denotes significant difference, while *n.s* indicates no significant difference

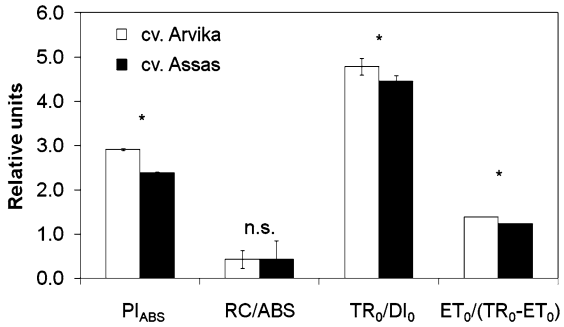

Fig. 5 Performance index (PI_{ABS}) and its components measured in green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves. All parameters are expressed in relative units. Columns are mean values and bars represent SE of 10 replicates. Significant difference was considered at $P < 0.05$ according to the Student's *t*-test; *asterisk* denotes significant difference, while *n.s* indicates no significant difference

4 Discussion

Winter peas usually start to develop in unfavourable environmental conditions with low temperatures and frosts. Under conditions of low temperature and short days, winter peas become more tolerant to frost (Munier-Jolain et al. 2010). Low temperature can induce downregulation of metabolic processes what may alter the balance between light capture and energy utilization (Allen and Ort 2001). Low temperatures (over 0 °C) was shown to reduce electron transport and even induce photoinhibition in pea plants that were not acclimated to such temperatures (Munier-Jolain et al. 2010). However, during

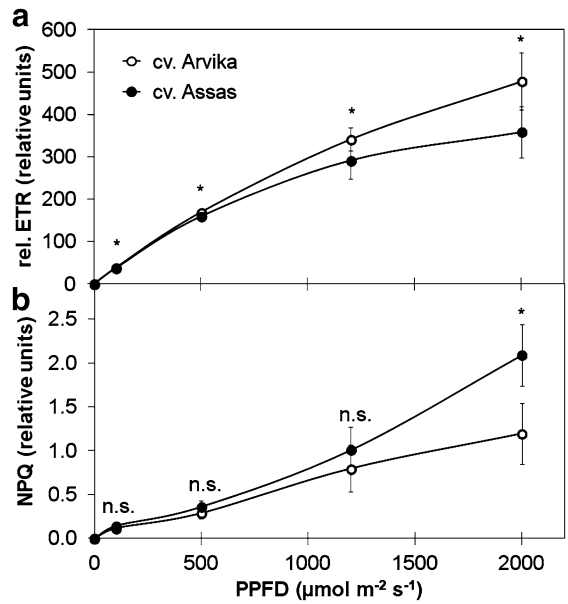

Fig. 6 Relative electron-transport rate (relETR; **a**) and non-photochemical quenching (NPQ; **b**) in green (cv. 'Arvika') and red (cv. 'Assas') pea (*Pisum sativum* L.) leaves. Dots are mean values and bars are SE of six replicates. Significant difference was considered at $P < 0.05$ according to the Student's *t*-test; *asterisk* denotes significant difference, while *n.s* indicates no significant difference

acclimation to cold conditions, plants accumulate soluble sugars that act as cryoprotectants and signalling molecules. In coordination with hormone signals and in the presence of light, sugars regulate the accumulation of anthocyanins (Das et al. 2011; Tanino et al. 2014). Thus, occurrence of anthocyanins in winter pea cv. 'Assas', is most likely to be an adaptation to endure suboptimal winter temperatures (Fig. 1b). On the other hand, cv. 'Arvika', being a spring cultivar, does not show such adaptation (Fig. 1a). However, both cultivars are characterized as tolerant to low temperatures. Anthocyanins accumulation is often described as undesirable feature since it is needed to invest more energy in their biosynthesis (Das et al. 2011). Nevertheless, during early stages of development, anthocyanin accumulation could have a photoprotective role (Landi et al. 2015). This could be the case in our investigation because red colouration of cv. 'Assas' disappeared later during plant development (flowering and seed development).

Localization of anthocyanins revealed its appearance only in red leaves of cv. 'Assas' at both, upper

and lower epidermis (Fig. 1d). It was suggested earlier that localization of anthocyanins in leaf is important in the context of anthocyanins function (Hatier and Gould 2009; Hughes et al. 2014). Anthocyanins in upper epidermis serve as a screen or internal filtering. They could reduce absorption of excessive irradiation and thus diminish the excitation pressure on photosynthetic apparatus. It was estimated that anthocyanins are able to absorb up to 70% of light entering the leaf and compete with light absorption by chlorophylls in the leaf. In that way, they are able to diminish the influence of excessive light on photosynthetic apparatus and reduce the risk of photoinhibition (Merzlyak et al. 2008; Ranjan et al. 2014; Solovchenko and Chivkunova 2011). During our investigation, it was observed that abaxial leaf surface of young cv. 'Assas' were facing upwards. Appearance of anthocyanins in lower epidermis is common in juvenile leaf stages that emerge with outward facing abaxial surface. When abaxial aonthocyanins are exposed to high light, their function is the protection of mesophyll because they attenuate the excessive light (Hughes and Smith 2007).

Absorption spectra measured in red leaves (Fig. 2) suggest that anthocyanins protect photosynthetic apparatus from UV irradiance since higher absorption was observed at lower wavelengths (360 nm). Hatier et al. (2013) reported that the presence of UV–A absorbing compounds, measured at 360 nm, were considerably higher in coloured *Ophiophogon* leaves that contained anthocyanins in comparison to green ones. Indeed, some anthocyanins have been associated with enhanced absorption capacity under UV-A wavelength (315–400 nm) (Landi et al. 2015; Takahashi and Badger 2011). It was also suggested that accumulation of anthocyanins compete with photosynthetic pigments for light capture at high photon flux and thus reduce photosynthetic efficiency (Manetas 2006). This could also be the case in our results since green pea leaves showed over 1.2 times higher effective quantum yield ($\Delta F/F_m'$) when compared to red ones after exposure to high light intensity (1200 and 2000 μmol m^{-2} s^{-1}). However, our results (Table 2) revealed that despite better absorption of green leaves in blue and red wavelengths under intermediate irradiance, chlorophyll content did not differ between red and green leaves.

The chlorophyll *a* transients revealed characteristic shapes in both leaf types (Fig. 3a). Both, F_0 and F_m did not differ between green and red leaves what is consistent with unchanged F_v/F_m values (Table 3). Both leaf types were able to reach maximal fluorescence intensity at 500–800 μmol m^{-2} s^{-1} since F_v/F_m in both leaf types were higher than 0.81 showing that this light intensity was enough to saturate PSII (Strasser et al. 2000). In addition, both leaf types showed fully functional PSII (values higher than 0.75) (Bolhar-Nordenkampf et al. 1989) measured in the field, indicating that photoinhibition did not occur. However, exposure of both leaf types to high light conditions (at 1200 and 2000 μmol m^{-2} s^{-1}) significantly decreased the effective quantum yield ($\Delta F/F_m'$). Although the photo inhibition was more severe in red leaves, the decrease in $\Delta F/F_m'$ (values below 0.75) suggested that both leaf types were photoinhibited.

The O-P normalized curves (Fig. 3b) showed increase at J and I steps and, in parallel, higher values of V_J and V_I in red leaves, suggesting more reduced PQ pool compared to green ones. Since V_J stands for the part of PSII RCs that close during single charge separation and it represents probability to move an electron further than Q_A^-, an increase in this parameter (Fig. 3c) indicates limited re-oxidation of Q_A in red leaves (Strasser et al. 2004). The V_I parameter gives us estimated measure of Q_B-non-reducing PSII centres, unable to reduce Q_B (Lebkuecher et al. 1999; Strasser et al. 2004). The Q_B-non-reducing RCs are described as inactive ones since electron transport between Q_A^- and Q_B is not functional. Therefore, the increase of V_I (Fig. 3c) in red leaves suggests an increased accumulation of reduced Q_A and plastoquinone, which cannot transfer electrons further than Q_A. The decrease in parameters describing electron transport, namely electron transport flux per active RC (ET_0/RC, Fig. 4) and electron transport beyond Q_A^- ($(ET_0/(TR_0 - ET_0)$, Fig. 5) confirmed reduced electron flow from Q_A^- to Q_B and further downstream. Also, decrease in $ET_0/(TR_0 - ET_0)$ along with reduced flux ratio of trapping and dissipation (TR_0/DI_0) contributed the most to decreased PI_{ABS} in red leaves (Fig. 5). The TR_0/DI_0 ratio describes the efficiency of the water-splitting complex on the donor side of PSII and a decrease of this parameter suggests structural damage to thylakoids (Kalaji et al. 2011; Pereira et al. 2000). Similar to our results, Ranjan et al. (2014) reported inhibition of electron transport at the PSII

donor side, electron transport per excited cross section and significantly low performance index of PSII in juvenile red *Jatropha curcas* L. leaves. On the other hand, these authors reported decreased trapping and absorption, which causes lower photosynthetic efficiency. In the present study, specific energy fluxes describing the distribution of energy through PSII at the reaction centre level (Fig. 4), absorption (ABS/RC), trapping (TR_0/RC) and dissipation (DI_0/RC) did not differ between green and red pea leaves. The ABS/RC measures the functional antenna size that supplies excitation energy to active RCs while TR_0/RC refers to the trapping of excited energy by active RCs. Therefore, any changes in ABS and TR_0 would proportionally change TR_0/ABS (F_v/F_m), the measure of trapping per total absorption or quantum yield for primary photochemistry (Yusuf et al. 2010). Our results indicate that reaction centres of both, red and green leaves were active and all absorbed energy was efficiently utilized to reduce Q_A, which is additionally supported by unaffected F_v/F_m values (Table 3).

Additional experiment with short-term high light treatment where saturating pulse method was used confirmed inhibited electron flow in red leaves since PSII driven relETR revealed decreased values at all measured light levels. There are several reports on lower relETR in anthocyanic leaves. Shao et al. (2008) observed lower ETR values in red *Arabidopsis* leaves. They suggested that plants deficient in anthocyanin synthesis are more sensitive to photooxidative disturbances and their ability to respond to stressful conditions were decreased. Landi et al. (2014) reported that a basil variety containing anthocyanins showed lower ETR compared to the green variety. Ranjan et al. (2014) reported decrease of ETR with the increase of PPFD in red juvenile *Jatropha curcas* L. leaves. All mentioned reports showed parallel increase in NPQ parameter. Non-photochemical quenching describes part of excitation energy harmlessly dissipated as heat (Müller et al. 2001). In the present study, only the highest light intensity (2000 µmol m^{-2} s^{-1}) induced significantly higher NPQ values, which is in accordance with recent report of Dewez and Perreault (2013) who revealed that NPQ is an important regulatory mechanism for anthocyanic leaves when light is in excess. In that case, anthocyanic epidermal layer serve as passive photo regulatory system for PSII. Based on that, our findings suggest that anthocyanins act as a photoprotection, shading chloroplasts from excessive light and thus, protecting them from the deleterious effects of high irradiance.

Considering lower electron transport and lower efficiency for absorbed light utilization in red leaves, it can be assumed that they had lower carbon assimilation compared to green leaves. It is known that accumulation of anthocyanins usually interferes with normal metabolism since it requires investment of more energy, which may decrease light energy capture, and ultimately reduce carbon assimilation (Burger and Edwards 1996; Das et al. 2011). Degradation of anthocyanins in vegetative tissues usually coincides with plant maturation. In the case of transient pigmentation, this is regulated and in vegetative tissues, where carbon assimilation has a primary function, prolonged anthocyanin accumulation is undesirable (Steyn et al. 2002). This might be the case in cv. 'Assas' because the red colouration disappears with the maturity in this plant.

We concluded that limited electron transport further than Q_A^- in red leaves of cv. 'Assas' seems to be the most important difference when compared to green leaves of cv. 'Arvika'. Both cultivars revealed equal light absorption efficiency and light trapping because both leaf types revealed equal levels of chlorophyll contents and had fully functional primary photochemistry (F_v/F_m). Lower capacity of Q_A re-oxidation and higher portion of Q_B-non-reducing reaction centres, characterized by inhibited ability of the electron transport between Q_A^- and Q_B decreased overall photosynthetic performance (PI_{ABS}) in cv. 'Assas'. Decreased PSII driven electron transport rate accompanied by higher non-photochemical quenching in leaves exposed to high irradiation confirmed our assumption that red pea leaves of cv. 'Assas' were not able to use light energy in photochemistry as efficient as green leaves of cv. 'Arvika'.

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