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Characterization of photosynthetic performance during natural leaf senescence in winter wheat: Multivariate analysis as a tool for phenotypic characterization

M. VILJEVAC VULETIĆ*⁺ and V. ŠPANIĆ**

Agrochemical Laboratory, Agricultural Institute Osijek, Južno pregrade 17, 31000 Osijek, Croatia*⁺
Department of Small Cereal Crops, Agricultural Institute Osijek, Južno pregrade 17, 31000 Osijek, Croatia**

Abstract

In the current research, we used chlorophyll fluorescence measurements and photosynthetic pigment content to evaluate onset and rate of the flag leaf senescence every 7 d beginning at the flowering stage (0 d after flowering, DAF) until late senescence stage (35 DAF) on three winter wheat field-grown varieties (Alka, Žitarka, and Olimpija) with the similar maturation time. OJIP curves implied that flag leaves of variety Alka detained the longest photosynthetic efficiency, while the earliest symptoms of senescence onset were indicated by positive L-band at 7 DAF and K-band at 14 DAF in variety Olimpija. A shift in I–P phase in the wheat flag leaves suggested the reduction of first acceptors in PSI, implying degradation of photosynthetic apparatus. Performance index (Plmax) proved to be the sensitive indicator of senescence; it decreased very early at 7 DAF in the flag leaves of variety Olimpija compared to Alka and Žitarka. Principal component analysis elucidated quantum yield of energy dissipation and dissipated energy flux per reaction centre as the earliest indicators of senescence in wheat flag leaves and thus showed that multivariate approach may be useful in detection of senescence onset. Sustainability indexes (SIs) corroborated the statistical significance results of evaluated parameters implying that SIs can be used as user friendly data analysis for screening purposes. Selection for functional stay-green trait could contribute to increasing crop yields.

Additional key words: fluorescence transient; grain filling; JIP-test; leaf maturation; photoinhibition; photosystem II.

Introduction

Senescence is natural process of aging of plant cells, tissues, and organs characterized as the last developmental stage in plants (Distelfeld et al. 2014). In wheat plants, the flag leaf senescence onset very often coincides with the grain-filling period, so it is the important factor in wheat production (Zhang et al. 2006) because approximately 30–50% of photosassimilates in wheat grain is coming from the flag leaf photosynthesis (Sylvester-Bradley et al. 1990). Leaf senescence is characterized by degradation of photosynthetic pigments and disassembling of photosynthetic apparatus as well as chloroplast protein degradation (Lu et al. 2001, Gregersen et al. 2008). These events negatively influence photosynthetic process, consequently resulting in disturbances in all aspects of the plant metabolism and physiology (Kalaji et al. 2018a). In previous research of Lu et al. (2001, 2002, 2003), during natural

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*Corresponding author; e-mail: marija.viljevac@poljinos.hr
Abbreviations: ABS/RC – absorption flux (of antenna chlorophylls) per reaction centre; Car – carotenoids; ChlF – chlorophyll a fluorescence; DAF – days after flowering; DI/R – dissipated energy flux per reaction centre; ETo/RC – electron transport flux (further than QA) per reaction centre; F0 – minimal fluorescence intensity (50 µs); FM – fresh mass; FV/FM – maximal variable fluorescence; OEC – oxygen-evolving complex; P0/RC – principal component analysis; Plmax – performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors; Plend – performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors; RC – reaction centre; RC/CS – density of reaction centres per cross section; REo/RC – electron flux reducing end electron acceptors at the PSI acceptor side per reaction centre; S1 – sustainability index; TRd/RC – trapping flux (leading to QA reduction) per reaction centre; W0/0 – relative variable fluorescence between O- and J-steps; W0/K – relative variable fluorescence between O- and K-steps; δ0 – probability that an electron from the electron transport chain is transferred to reduce end electron acceptors at the PSI acceptor side; φQ0 – quantum yield of energy dissipation at t = 0; φ0 – maximum quantum yield for primary photochemistry at t = 0; φe0 – quantum yield for reduction of end electron acceptors at the PSI acceptor side; ψ1 – probability that a trapped exciton moves an electron into the electron transport chain beyond QA.
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flag leaf senescence, photosynthetic performance of the field-grown wheat plants was investigated. By the use of modulated chlorophyll (Chl) fluorescence technique, they found that PSII apparatus during senescence progressing remained functional, but downregulated under the steady state of photosynthesis. They associated such a down-regulation with the closure of PSII centres and an enhanced xanthophyll cycle-related thermal dissipation in the PSII antennae. PSII downregulation and activation of xanthophyll cycle occurred after degradation of Chl, followed by neoxanthin and β-carotene pigments, accompanied by increased Chl a/b ratio at one side and lutein and xanthophyll pigments maintenance at the other side. 

Zhang et al. (2006) found that later onset and slower rate of senescence in wheat variety NM9 grown in the field may be responsible for its higher grain yield than that in variety NM8 ageing earlier. Similar results were found by Yang et al. (2007) who showed that the higher photosynthetic capacity can accumulate more dry matter in the flag leaf of wheat hybrid compared to its parents, and might be the physiological basis for the higher grain yield.

Photosynthesis deals with energy flow through the plant, starting with light absorption, its conversion to chemical energy, and dissipation of unused energy trough thermal dissipation or/and Chl fluorescence (ChlF). Therefore, ChlF, as concomitant process to energy absorption, conversion, and thermal dissipation, can be used for estimation of photosynthetic efficiency (Maxwell and Johnson 2000). Recently, modern fluorimeters, which record emitted fluorescence in high frequency, made ChlF technic a user friendly scientific tool because it performs fast and noninvasive analysis and gives powerful data about photosynthetic process. Such fluorimeters induce ChlF by amplification of short pulse (1-s lasting) of strong actinic light on the dark-adapted leaf. Recorded fluorescence data (118 data in 1 s) present fast polyphasic fluorescence transient and this kinetics (OJIP kinetics) is basis for the photosynthetic efficiency interpretation (Kalaji et al. 2014). Analysis of OJIP kinetics, called the JIP-test (Strasser et al. 2004), gives us numerous structural and functional parameters that quantify photosynthetic performance through PSII complexes (Yusuf et al. 2010).

Important advantage of OJIP kinetics is that it can capture delicate alterations in photosynthetic performance which other techniques based on ChlF measurement cannot. JIP-test calculations describe specific energy fluxes, phenomenological fluxes, and performance indexes (Strasser et al. 2004) altogether with quantum yields, photochemical and nonphotochemical quenching obtained by saturation pulse method (Maxwell and Johnson 2000). Furthermore, the shape of characteristic OJIP curve can be changed under nonoptimal conditions (biotic or abiotic stress) for photosynthesis, in the way that unusual shift of fluorescence can be observed at 300 µs (K-band), 150 µs (L-band) as well as shift in 1-step (about 30 ms) (Yusuf et al. 2010, Kalaji et al. 2018a, Pavlović et al. 2019). Above mentioned facts enable to detect alterations in photosynthetic process, while the most frequently used parameters, such as maximal quantum yield of PSII (Fv/Fm) and performance index (PI), stay still unchanged. Since ChlF is fast, noninvasive, informative, and cheap technic, it is widely eligible tool for screening of breeding material for different purposes (Kovačević et al. 2017, Kalaji et al. 2018b, Galić et al. 2019, Rapacz et al. 2019).

ChlF transient completed by JIP-test is very informative and might give a detailed explanation of downregulation of photosynthesis during the senescence process. According to our best knowledge, such investigations on the wheat flag leaves are still missing. Our main goal was to identify one or more JIP parameters sensitive enough for the early detection of senescence onset because it could be an important tool for wheat breeders who are targeting selection for ‘stay-green’ genotypes. Furthermore, the main objective of this study focused on the onset of the senescence in three winter wheat varieties with similar maturation time through chlorophyll fluorescence transient followed by JIP-test and concentrations of photosynthetic pigments. Above mentioned analyses provided numerous parameters, so we hypothesised that the use of multivariate method of data analysis should elucidate the most informative parameter(s) for use in wheat breeding selection. Furthermore, wheat varieties could be differentiated with explanation of relations among the investigated parameters during the flag leaf maturation and senescence.

Materials and methods

Plant material and experimental design: The experiment was conducted at Agricultural Institute Osijek (AIO), Osijek, Croatia, on three winter wheat (Triticum aestivum L.) field-grown varieties (Alka, Žitarka, and Olimpija) with similar maturation time in vegetation season 2016–2017. The varieties were sown during October in 2016 in 7.56 m² randomized plots using a Seedmatic seeding machine (Hege, Germany) at the experimental field (45°32’N, 18°44’E). Seed density was 330 seeds m⁻² for all varieties, where each variety was replicated in two plots. To meet the winter wheat plant nutrient requirements, fertilization was done during the study (N:P:K, 120:80:120 kg ha⁻¹). Pesticides and herbicides were used as necessary to minimise the effects of pests and weeds. The mean annual temperature during the vegetation season was 10.0°C with the sum of annual precipitation during this period of 481.5 mm (Fig. 1). The date of flowering (growth stage 65, Zadoks et al. 1974) was determined when plants shed at least 50% of anthers of the spike throughout each plot. Measurements and samplings were performed in 7-d intervals from flowering to 35 d after flowering (DAF).

Measurement of the fast Chl a fluorescence transient: Measurements of ChlF were made on the one fully expanded flag leaf of five different plants in each plot using a plant efficiency analyser (Handy PEA, Hansatech Instruments Ltd., Norfolk, UK). Flag leaves were dark-adapted for 30 min using special leaf clips. ChlF transient was induced by red actinic light [wavelength at peak 650 nm; 3,200 μmol(phon) m⁻² s⁻¹] and 1 s of transient fluorescence was recorded. Fluorescence signals have been collected from 10 μs up to 1 s with data acquisition every 10 μs for the first 300 μs, then continued every 100 μs.
up to 3 ms, and later every 1 ms, 118 points within 1 s in total. ChlF transient data were demonstrated as OJIP curves and used for calculation of JIP-test parameters (Table 1S, supplement).

**Analysis of Chl fluorescence data:** The OJIP transients were double-normalized between O- (50 μs) and P-steps and presented as the relative variable fluorescence \[ W_{OP} = \frac{(F_t - F_0)}{(F_P - F_0)} \] on a logarithmic scale. Native fluorescence induction curves were also presented. Furthermore, normalization between O- and K- (300 μs) steps detected L-band (150 μs) which was demonstrated as variable fluorescence \[ W_{OK} = \frac{(F_t - F_0)}{(F_K - F_0)} \] where \( (W_{OK})_{ref} \) represented reference values obtained from measurements at 0 DAF. Similarly, normalization between O- and J- (2 ms) steps detected K-band (300 μs) which was demonstrated as variable fluorescence \[ W_{OJ} = \frac{(F_t - F_0)}{(F_J - F_0)} \] on the linear time scale plotted with difference kinetics \( \Delta W_{OJ} = W_{OJ} - (W_{OJ})_{ref} \), where \( (W_{OJ})_{ref} \) represented referent values obtained from measurements at 0 DAF (Strasser et al. 2004, Yusuf et al. 2010).

The particular data points of ChlF transients were used to calculate JIP-test-derived parameters according to Strasser et al. (2000). The analysed parameters and their equations are described in Table 1S.

**Determination of photosynthetic pigments content:** After measurement of the ChlF, the same flag leaves were taken, pooled together, and stored at –80°C until further analysis. Leaf tissue was homogenized into a fine powder by liquid nitrogen with addition of magnesium hydroxide carbonate and five replicates were separated (0.1 g) from each composed sample. Photosynthetic pigments were extracted with the cold absolute acetone and then reextracted several times until plant tissue was completely uncoloured. The concentrations of Chl \( a \), Chl \( b \), and carotenoids (Car) were determined spectrophotometrically (Specord 200, Analytik Jena, Germany) at 470, 661.6, and 644.8 nm according to Lichtenthaler (1987) and expressed as mg g\(^{-1}\) of fresh mass. The Chl \( a/b \) was calculated.

**Statistical analysis:** Statistical differences between all parameters in three winter wheat varieties at six time points (0, 7, 14, 21, 28, and 35 DAF) were analysed using analysis of variance followed by post hoc Fisher’s Least Significant Difference (LSD) test. Data presented in the text, figures, and tables are means ± SE of ten biological replicates. Differences were considered significant at \( P<0.05 \). Correlations among JIP parameters, photosynthetic pigments and winter wheat varieties were explored by principal component analysis (PCA) in order to distinguish parameters, which could be indicators of the senescence onset. The analyses were performed on ten cases for each sampling point (DAF) in Statistica 7.0 software (Statsoft Inc., Tulsa, OK, USA).

**Results**

The first visible symptoms of senescence were seen as yellowing of apical parts of flag leaves at 7 DAF in Olimpija (Fig. 2). In flag leaves of varieties Alka and Žitarka, the first symptoms of senescence occurred at 14 and 21 DAF, respectively, exhibited as sporadic yellow blur on the whole leaf surface.

**Chl \( a \) fluorescence:** Variable fluorescence curves were constructed to find changes in the photosynthetic performance of winter wheat varieties during natural leaf
senescence (Fig. 3). The first visible changes between variable fluorescence curves occurred at I-step starting already at 7 DAF in the flag leaves of Alka and the same trend continued until the end of the experiment accompanied by alterations in the whole OJIP curve which occurred at 28 and 35 DAF (Fig. 3A). In the flag leaves of variety Žitarka (Fig. 3C), the first visible changes on OJIP curve were observed in I-step at 21 DAF followed by alterations in the whole OJIP curve starting at 28 DAF. Flag leaves of variety Olimpija during experimental period revealed alterations in the whole OJIP curve starting already at 7 DAF (Fig. 3E). Fluorescence induction curves (Fig. 3 B,D,F) revealed distinct differences between varieties. Shape of OJIP induction curve at 28 DAF showed that in Alka, photochemistry remained functional, while in Žitarka and Olimpija, flattening of fluorescence induction curve occurred, more markedly in Olimpija. Fluorescence induction curves were flattened in all three varieties at 35 DAF. A clear differences in L-bands (Fig. 4A,C,E) and K-bands (Fig. 4B,D,F), generated from the normalized O–K and O–J curves, were found within varieties. The first negative effects of natural senescence on photosynthesis were seen as the positive L-bands in the flag leaves of variety Olimpija at 7 DAF (Fig. 4E) accompanied with positive K-bands at 14 DAF (Fig. 4F). Other two varieties reacted similarly but the L-bands prolonged time to positive level at 28 DAF; while K-bands in Žitarka were negative until 28 DAF, in Alka, this was until 14 DAF.

The significant differences were found between varieties in most of the JIP-test parameters during natural flag leaf senescence of winter wheat varieties (Fig. 5; Table 2S, supplement) as well as between the time of measurements. JIP-test parameters shown in radar plots were normalized to enable the comparison of the variables measured at a different scale. Values at the radar plot presented percentage of values obtained for measurements at 0 DAF. At the 7 DAF, no significant differences were between fluorescence indices (F₀ and Fᵥ), quantum yields and efficiencies (φD₀, φP₀, φR₀, φE₀, ψE₀, and δR₀), specific energy fluxes, and phenomenological fluxes (ABS/RC, TR₀/RC, ET₀/RC, DL/R, RE/R, and RC/CS₀), and performance indexes (PlABS and Plmax) in the flag leaves of Alka (Fig. 5A). The first significant changes

![Fig. 3. Double O–P normalized OJIP transients (A,C,E) and native fluorescence induction curves (B,D,F) in the flag leaves of winter wheat varieties Alka (A,B), Žitarka (C,D) and Olimpija (E,F) at 0, 7, 14, 21, 28, and 35 d after flowering (DAF). Each curve presents average kinetics of ten replicates.](image-url)
appeared at 14 DAF as the decrease of $\varphi_{R0}$, $\delta_{R0}$, $RE_0/RC$, and $PI_{total}$ compared to 0 DAF. All ChlF parameters remained significantly at the same level until 28 DAF, when significant decreases were found in $\varphi_{R0}$, $RE_0/RC$, $PI_{ABS}$, and $PI_{total}$. At 35 DAF, all investigated parameters were significantly altered with exception of $F_{m}$ which remained at the same level.

ChlF-derived parameters in the flag leaves of winter wheat variety Žitarka changed already at 7 DAF, compared to 0 DAF (Fig. 5B), namely $F_{v}$, $\varphi_{R0}$, $\delta_{R0}$, $RE_0/RC$, $RC/CS$, and $PI_{total}$. At 14 DAF, only $PI_{ABS}$ decreased, while at 21 DAF, the larger significant alterations started to be seen as decrements of $F_{v}$, $\varphi_{R0}$, $\delta_{R0}$, $RE_0/RC$, $PI_{ABS}$, and $PI_{total}$. All traced ChlF parameters significantly changed at 28 DAF in the way that most parameters decreased, except increase of $\varphi_{D0}$, $ABS/RC$, $TR_{v}/RC$, and $DL_{v}/RC$. Similar significant alteration also occurred at 35 DAF.

Several of the ChlF parameters in the flag leaves of winter wheat variety Olimpija decreased already at 7 DAF, namely $F_{v}$, $\varphi_{R0}$, and $PI_{ABS}$ (Fig. 5C). Following significant changes appeared at 14 DAF as diminution in $\varphi_{R0}$, $RE_0/RC$, and $PI_{total}$ and decrement continued at 21 DAF in $\varphi_{E0}$, $\psi_{E0}$, $\delta_{R0}$, $RC/CS$, and $PI_{ABS}$. At 35 DAF, all traced ChlF parameters significantly altered with exception of $\varphi_{R0}$ and $PI_{total}$ which remained at the same level. Parameters $\varphi_{D0}$, $ABS/RC$, $TR_{v}/RC$, and $DL_{v}/RC$ increased, while others decreased, similarly as it was in the flag leaves of varieties Alka and Žitarka.

**Photosynthetic pigments:** Chl $a$ concentration increased at 7 DAF in the flag leaves of Alka and Žitarka, but started decreasing continuously from 14 DAF till the end of experiment (35 DAF) (Fig. 6A, Table 2S). In the flag leaves of Olimpija, a significant diminution of Chl $a$ started at 14 DAF and it continued during the experiment. In variety Žitarka, a similar trend was observed also for Chl $b$ (Fig. 6B), while in the flag leaves of varieties Alka and Olimpija, the concentration of Chl $b$ remained unchanged until 28 DAF and 21 DAF, respectively, then started decreasing and diminution continued until the end of the experiment. Carotenoids (Car) in the flag leaves of variety Alka were
at the same level until 28 DAF when they significantly decreased and the same trend continued till 35 DAF (Fig. 6C). In the flag leaves of Žitarka and Olimpija, the first significant change in the Car content was found at 14 DAF, seen as diminution which continued until 35 DAF. Values of Chl \( a/b \) (Fig. 6D) in the leaves of Alka increased significantly at 35 DAF, while in varieties Žitarka and Olimpija at 21 DAF and 28 DAF, respectively; after that it continued increasing until the end of experiment.

Sustainability index (SI): The SI of photosynthetic pigment contents and photosynthetic parameters of the winter wheat varieties Alka \((A)\), Žitarka \((B)\) and Olimpija \((C)\) at 0, 7, 14, 21, 28, and 35 d after flowering (DAF). Each data presents average value of ten replicates normalized and shown as percentage of values obtained at 0 DAF, enabling the comparison of variables measured on different scales. Raw data shown in radar plots and statistical differences obtained by ANOVA followed by Fisher’s LSD test \((P<0.5)\) are presented in Table 2S. F\(_{0}\) – minimal fluorescence intensity \((50 \mu s)\); F\(_{v}\) – maximal variable fluorescence; \( \varphi_{D0} \) – quantum yield of energy dissipation at \( t = 0 \); \( \varphi_{P0} \) – maximum quantum yield for primary photochemistry at \( t = 0 \); \( \varphi_{E0} \) – quantum yield of electron transport at \( t = 0 \); \( \psi_{E0} \) – probability that a trapped exciton moves an electron into the electron transport chain beyond Q\(_{A}\); \( \delta_{R0} \) – probability that an electron from the electron transport chain is transferred to reduce end electron acceptors at the PSI acceptor side; \( \text{ABS/RC} \) – absorption flux (of antenna chlorophylls) per RC; \( \text{TR} \) – trapping flux (leading to Q\(_{A}\) reduction) per RC; \( \text{ET} / \text{RC} \) – electron transport flux (further than Q\(_{A}\)) per RC; \( \text{DL} / \text{RC} \) – dissipated energy flux per RC; \( \text{RE} / \text{RC} \) – electron flux reducing end electron acceptors at the photosystem I (PSI) acceptor side per RC; \( \text{PC/CS} \) – density of reaction centres per cross section; \( \text{PI}_{\text{ABS}} \) – performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors; \( \text{PI}_{\text{total}} \) – performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors.

**Fig. 5.** Radar plots of structural and functional JIP-test parameters in the flag leaves of winter wheat varieties Alka \((A)\), Žitarka \((B)\) and Olimpija \((C)\) at 0, 7, 14, 21, 28, and 35 d after flowering (DAF). Each data presents average value of ten replicates normalized and shown as percentage of values obtained at 0 DAF, enabling the comparison of variables measured on different scales. Raw data shown in radar plots and statistical differences obtained by ANOVA followed by Fisher’s LSD test \((P<0.5)\) are presented in Table 2S. F\(_{0}\) – minimal fluorescence intensity \((50 \mu s)\); F\(_{v}\) – maximal variable fluorescence; \( \varphi_{D0} \) – quantum yield of energy dissipation at \( t = 0 \); \( \varphi_{P0} \) – maximum quantum yield for primary photochemistry at \( t = 0 \); \( \varphi_{E0} \) – quantum yield of electron transport at \( t = 0 \); \( \psi_{E0} \) – probability that a trapped exciton moves an electron into the electron transport chain beyond Q\(_{A}\); \( \delta_{R0} \) – probability that an electron from the electron transport chain is transferred to reduce end electron acceptors at the PSI acceptor side; \( \text{ABS/RC} \) – absorption flux (of antenna chlorophylls) per RC; \( \text{TR} \) – trapping flux (leading to Q\(_{A}\) reduction) per RC; \( \text{ET} / \text{RC} \) – electron transport flux (further than Q\(_{A}\)) per RC; \( \text{DL} / \text{RC} \) – dissipated energy flux per RC; \( \text{RE} / \text{RC} \) – electron flux reducing end electron acceptors at the photosystem I (PSI) acceptor side per RC; \( \text{PC/CS} \) – density of reaction centres per cross section; \( \text{PI}_{\text{ABS}} \) – performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors; \( \text{PI}_{\text{total}} \) – performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors.

306
PHOTOSYNTHETIC PERFORMANCE DURING LEAF SENESCENCE IN WHEAT

experimental point separately (0, 7, 14, 21, 28, and 35 DAF) with the aim (1) to find parameters, which indicate the onset of senescence, (2) to separate wheat varieties according senescence onset, and (3) to explain relations among the parameters at flag leaf maturation and senescence (Fig. 7).

At 0 DAF (Fig. 7A), parameters were grouped as follows: specific energy fluxes (ABS, TR, ET, DI, RE per RC) at the right side, and efficiencies and performance indexes at the left side of biplot. Variety Žitarka highly correlated with Chl a, Car, and Chl a/b content and parameters F0 and DI/RC. Varieties Alka and Olimpija were located at the opposite side of biplot, close to parameters PiABS, φD0, and φE0. Varieties were distinctly separated at 7 DAF (Fig. 7B); whereas Žitarka correlated with specific energy fluxes and Chls, Alka with parameters describing dissipation and electron acceptors at the PSI acceptor side, and Olimpija with PiABS, Car, Chl a/b, RC/CS0, and parameters describing electron transport. At 14 and 21 DAF (Fig. 7C,D), Alka was located at the opposite side of biplot, compared to Olimpija and Žitarka, and highly correlated with φD0 at 14 DAF and with RC/CS0, Chl a and b, Car, and PiABS at 21 DAF. At the other side, Žitarka and Olimpija correlated with parameters describing dissipation and electron acceptors at the PSI acceptor side at 14 DAF and dissipation at 21 DAF. PCA biplot for 28 DAF (Fig. 7E) is crucial for understanding of senescence onset because experimental parameters and varieties were divided by their high correlation in the way that variety Olimpija was isolated at the left side of biplot close to parameters Chl a/b, φD0, DI/RC, and ABS/RC. Žitarka and Alka were positioned at the right side of biplot, whereas Žitarka was close to performance indexes, parameters describing electron transport and electron acceptors at the PSI acceptor side, while Alka correlated with φD0, F0, FV, Chl a and b. At 35 DAF (Fig. 7F), variety Alka was in the correlation with Chl a and b, Car and F0, while Žitarka and Olimpija correlated with Chl a/b and parameters describing electron acceptors at the PSI acceptor side.

Discussion

In the wheat, recent studies of senescence focused on the flag leaf because it has been proven that flag leaf is the main source of assimilates during the grain-filling period which coincides with the onset of senescence (Wardlaw 1990, Zhang et al. 2006). Flag leaf senescence can be visualised as a leaf yellowing due to Chl loss but numerous additional molecular and biochemical processes contribute to the senescence syndrome as well (Distelfeld et al. 2014). The most significant syndrome of senescence is the decrease of photosynthetic capacity caused by disintegration of photosynthetic apparatus resulting from degradation of pigments and proteins (Gregersen et al. 2008). In the present study, changes in photosynthetic efficiency and photosynthetic pigment contents in the flag leaves of three winter wheat varieties grown in the field were investigated during period from the flowering stage.
Table 1. Sustainability index (SI) of photosynthetic pigment contents and photosynthetic parameters in flag leaves of varieties Alka, Žitarka, and Olimpija at 7, 14, 21, 28 and 35 d after flowering (DAF), compared to data taken at the time of flowering (0 DAF); SI = (the values at 7, 14, 21, 28 or 35 DAF)/(the values at 0 DAF). Chl \( a \) – chlorophyll \( a \); Chl \( b \) – chlorophyll \( b \); Car – carotenoids; \( F_0 \) – minimal fluorescence intensity (50 µs); \( F_v \) – maximal variable fluorescence; \( \varphi_D \) – quantum yield of energy dissipation at \( t = 0 \); \( \varphi_P \) – maximum quantum yield for primary photochemistry at \( t = 0 \); \( \eta \) – quantum yield for reduction of end electron acceptors at the PSI acceptor side; \( \delta \) – probability that an electron from the electron transport chain is transferred to end electron acceptors at the PSI acceptor side; ABS/RC – absorption flux (of antenna chlorophylls) per RC; TR/RC – trapping flux (leading to \( Q_e \) reduction) per RC; ETR/RC – electron transport flux (further of \( Q_e \) ) per RC; DI/RC – electron flux reducing end electron acceptors at the photosystem I (PSI) acceptor side per RC; RC/CS – density of reaction centres per cross section; \( \psi_{ABS/PSI} \) – performance index (potential) for energy conservation from exciton to the reduction of inter-system electron acceptors; \( \psi_{ABS/PSI} \) – density index (potential) for energy conservation from exciton to the reduction of PSI end acceptors.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Alka</th>
<th>Žitarka</th>
<th>Olimpija</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Chl } a )</td>
<td>1.081</td>
<td>1.012</td>
<td>1.054</td>
</tr>
<tr>
<td>( \text{Chl } b )</td>
<td>1.024</td>
<td>0.956</td>
<td>0.882</td>
</tr>
<tr>
<td>( \text{Car} )</td>
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<td>1.020</td>
<td>1.111</td>
</tr>
<tr>
<td>( \text{Chl } a/b )</td>
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<td>1.045</td>
<td>1.174</td>
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<td>( F_0 )</td>
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<td>1.005</td>
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</tr>
<tr>
<td>( F_v )</td>
<td>1.120</td>
<td>1.002</td>
<td>0.918</td>
</tr>
<tr>
<td>( \varphi_D )</td>
<td>0.966</td>
<td>0.949</td>
<td>1.068</td>
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<tr>
<td>( \varphi_P )</td>
<td>1.007</td>
<td>1.018</td>
<td>1.004</td>
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<td>( \varphi_{PSI} )</td>
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<tr>
<td>( \psi_{ABS/PSI} )</td>
<td>1.003</td>
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<td>1.014</td>
</tr>
<tr>
<td>( \delta )</td>
<td>0.996</td>
<td>0.994</td>
<td>1.009</td>
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<td>( \psi_{ABS/PSI} )</td>
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<td>0.799</td>
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<td>( \text{ETR/RC} )</td>
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<td>( \text{DI/RC} )</td>
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<td>0.939</td>
<td>0.967</td>
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<td>( \text{RE/RC} )</td>
<td>0.885</td>
<td>0.831</td>
<td>0.733</td>
</tr>
<tr>
<td>( \text{RC/CS} )</td>
<td>1.077</td>
<td>0.981</td>
<td>1.005</td>
</tr>
<tr>
<td>( \psi_{ABS/PSI} )</td>
<td>1.068</td>
<td>1.061</td>
<td>1.074</td>
</tr>
<tr>
<td>( \psi_{ABS/PSI} )</td>
<td>0.864</td>
<td>0.702</td>
<td>0.610</td>
</tr>
</tbody>
</table>
until senescence (35 DAF). Although three investigated wheat varieties were similar according to their maturation time, the present study revealed delicate distinctions in their flag leaf photochemistry by analysis of OJIP kinetics.

Numerous studies proved that the shape of OJIP curve is strongly modified under different stresses (Mehta et al., 2010). The results of two-dimensional principal component analysis (2D-PCA) of structural and functional JIP-test parameters and photosynthetic pigments in the flag leaves of winter wheat varieties Alka, Žitarka and Olimpija obtained at 0 (A), 7 (B), 14 (C), 21 (D), 28 (E), and 35 (F) days after flowering (DAF) are illustrated in Figure 7. Chl $a$ – chlorophyll $a$; Chl $b$ – chlorophyll $b$; Car – carotenoids; $F_0$ – minimal fluorescence intensity (50 µs); $F_v$ – maximal variable fluorescence; $\varphi_D$ – quantum yield of energy dissipation at $t = 0$; $\varphi_R$ – maximum quantum yield for primary photochemistry at $t = 0$; $\varphi_R^e$ – quantum yield for reduction of end electron acceptors at the PSI acceptor side; $\varphi_E$ – quantum yield of electron transport at $t = 0$; $\psi_E$ – probability that a trapped exciton moves an electron into the electron transport chain beyond $Q_A$; $\delta_R$ – probability that an electron from the electron transport chain is transferred to reduce end electron acceptors at the PSI acceptor side; ABS/RC – absorption flux (of antenna chlorophylls) per RC; TR/RC – trapping flux (leading to $Q_A$ reduction) per RC; ET/RC – electron transport flux (further than $Q_A$) per RC; DI/RC – dissipated energy flux per RC; RE/RC – electron flux reducing end electron acceptors at the photosystem I (PSI) acceptor side per RC; PI$_{ABS}$ – performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors; PI$_{total}$ – performance index (potential) for energy conservation from exciton to the reduction of PSI end acceptors.
et al. 2010, Yusuf et al. 2010, Fan et al. 2014, Kalaji et al. 2018a, Paunov et al. 2018), in phenotyping mutants in contrast to wild types (Lepeduk et al. 2009, Yusuf et al. 2010), during diurnal changes in the photochemistry (Mlinarić et al. 2017) or during the growing season (Sitko et al. 2019). In the current study, senescence onset induced shifts in all parts of OJIP curve. The increase in O–J phase started at 28 DAF in Alka, much earlier in Olimpija and Žitarka, and consequently reducing reflection of the acceptor side of PSII (Kalaji et al. 2016). Better understanding of changes in O–J phase and significant differences in photochemistry of experimental wheat varieties have been obtained by visualisation of two additional steps (K- and L-band) (Yusuf et al. 2010). The difference kinetics $\Delta W_{OJ}$ revealed positive L-bands in all measurements on the leaves of Olimpija indicating low energetic connectivity and stability of PSII units. This means that onset of senescence in Olimpija started with a partial disruption of thylakoid structures as evidenced in decrease of energy transfer between PSII units, similarly as Paunov et al. (2018) found for Cd and Zn toxic impact on durum wheat leaf photochemistry. Positive L-bands in the leaves of Alka and Žitarka were found at the later growth stages. In addition, K-band revealed by $\Delta W_{OJ}$ difference kinetics in Olimpija was shifted in positive way already at 14 DAF, earlier than that of two other varieties indicating that Alka and Žitarka detained functional PSII antenna longer than Olimpija, where imbalance between electron donor and electron acceptor side of PSII occurred and caused impaired oxygen-evolving complex (OEC) function. In the recent literature, the negative K-band is widely used as a sign of tolerance to various stresses (Mathur et al. 2011, Fan et al. 2014, Krüger et al. 2014, Begović et al. 2016). Similar positive K- and L-band occurred in the leaves of quick-leaf senescence compared to stay-green Zea mays L. inbred lines when senescence was artificially induced by ethephon (Zhang et al. 2012) suggesting that appearance of positive K- and/or L-band can be characterised as a senescence onset marker. J–I phase is characterised by reduction of the intersystem electron carriers, e.g., secondary electron acceptor Q$_b$, plastoquinone, cytochrome, and plastoeryxin (Yusuf et al. 2010, Kalaji et al. 2016). Increase in J–I transient during the experiment was visible in the flag leaves of Olimpija earlier than that in other varieties which supported previous evidence of senescence onset in the flag leaves of Olimpija. In the flag leaves of Alka and Žitarka at 7 DAF, the increase of fluorescence in I-step and positive peak of I–P transient was found which could be connected with a smaller pool of plastoquinone and/or PSI end electron acceptors per active PSI RC (Kalaji et al. 2016, Paunov et al. 2018), resulting in delay in further electron transfer among electron transport chain. Failure of PSI to oxidise reduced plastoquinone leads to an accumulation of the reduced states which is typical reaction to heat stress in wheat (Mathur et al. 2011). Delicate differences in Chl fluorescence changes, seen on native fluorescence curve during experimental period in the flag leaves of wheat varieties, suggested that first symptoms of alterations in photochemistry occurred in Olimpija, followed by Žitarka and Alka. Flattened curves at 35 DAF were almost linear, suggesting a complete disassembly of photosynthetic apparatus and onset of photosynthesis (Sitko et al. 2019).

In order to quantify the changes in light absorption, its utilisation, and conversion to chemical energy and to analyse fluorescence transient, JIP-test was conducted and parameters shown on radar plots gave the cleaner picture of senescence onset in different wheat varieties. The most prominent changes of JIP parameters during natural senescence were visible in the flag leaves of Olimpija which was in accordance with shifts in its OJIP curves and early appearance of positive K- and L-bands although statistically significant difference in JIP parameters was found only for $P_{\mathrm{tot}}$ at 7 DAF. Subsequent significant alteration was found in parameters explaining the state of end electron acceptors at the PSI acceptor side ($\phi_{\mathrm{RE}}$ and $\phi_{\mathrm{ET}}$) suggesting that although other JIP parameters did not significantly disturbed, appearance of positive K- and L-bands was early indicator of impaired photochemistry in the senescence process. Furthermore, significant decline in $P_{\mathrm{tot}}$ performance index for overall energy flow from exciton to the reduction of PSI end acceptors, coincided with the decrease of parameters $\phi_{\mathrm{ET}}$ and $\phi_{\mathrm{RE}}$. Pavlović et al. (2019) also found that under the salinity stress in Brassica species, the most significant impact on $P_{\mathrm{tot}}$ had the parameter which reflects the efficiency of processes involving PSI and its ability to reduce its end acceptors [$\mathrm{RE}_{\mathrm{sat}} / (\mathrm{ET}_{\mathrm{sat}} - \mathrm{RE}_{\mathrm{sat}})$]. However, the most used parameter in ChlF, $\phi_{\mathrm{F0}}$, was not affected by senescence process until senescence symptoms become pronounced. $\phi_{\mathrm{F0}}$ decreased at 28 DAF in Olimpija, compared to other varieties where decrease of $\phi_{\mathrm{F0}}$ occurred at 35 DAF. Performance indexes ($P_{\text{ABS}}$ and $P_{\text{tot}}$) decreased earlier than $\phi_{\text{F0}}$. $P_{\text{tot}}$ proved to be much more sensitive than $P_{\text{ABS}}$ under senescence conditions. Sitrbet et al. (2018) emphasised that sensitivity of $P_{\text{ABS}}$ and $P_{\text{tot}}$ is different depending on factors, which caused photochemical alterations in photosystem. Panda and Sarkar (2013) found that $\phi_{\text{F0}}$ remained unchanged with the progression of senescence in rice flag leaves which supported previous findings by Falque et al. (2009) and Lu et al. (2003). In addition, $P_{\text{ABS}}$ appeared to be sensitive indicator of senescence onset and progression in rice leaves (Zhang et al. 2010, Panda and Sarkar 2013). In the current research, the highest increase was observed in parameters describing light absorption (ABS/RC) and dissipation of unutilized energy ($\phi_{\text{D0}}$ and $D_{\text{L0}}$) which is characteristic in senescence process as well as under stress conditions. Lu et al. (2001, 2002) showed that in the field conditions 25 d after anthesis, photochemical quenching of wheat flag leaves decreased significantly, while at the same time, nonphotochemical quenching increased to dissipate surplus captured energy suggesting downregulation of PSII and activation of photoinhibition process. Photoinhibition is following process when downregulation of PSII occurs due to disassembly of photosynthetic apparatus. The major loss of Chls, seen as the decline in their concentrations during the experimental period, and increase in Chl a/b ratio implied alterations in the photosynthetic pigments stoichiometry during leaf senescence (Lu et al. 2001).
Despite earlier Chl loss (at 14 DAF), Chl a/b remained unchanged until 28 DAF as well as majority of JIP parameters, suggesting that ChlF is remarkably insensitive to changes of the leaf Chl content until Chl a/b stayed unaffected (Dinç et al. 2012). According to our results, Chl a was more affected than Chl b and taken together with earlier decline of Car in the flag leaves of Olimpija (21 DAF), it suggests that photoinhibition occurred in this variety earlier than that in other two varieties. The decline in Car content implied that the xanthophyll cycle was not activated properly to protect PSII from photoinhibition (Jahns and Holzwarth 2012).

Among all results, it is hard to distinguish importance of any certain parameter. Hence, we calculated sustainability indexes (SIs) per sampling points to alleviate result interpretation. SIs corroborated with the results of statistical significances (Table 1. vs. Table 2S) implying that SIs can be used as user friendly data analysis for screening purposes of wheat material as it was concluded in previous research of Panda and Sarkar (2013).

Analyses of OJIP curve and JIP-test assigned numerous findings and conclusions, with the requirement of a significant knowledge and understanding of photosynthetic process in details. However, numerous parameters obtained by ChlF are confusing with the need of multivariate approach in statistical analysis. JIP-test parameters are calculated on the basis of fluorescence transient curve points and some of them were in high correlation because of their mathematical expressions (e.g., $q_{00}$ and $q_{0a}$). Thereby, multivariate statistical method such as PCA is method of choice because it is based on calculation of new complex variables that reflect maximal changes in the experimental data set. Usually, first two principal components explain the majority of variations which in our case were induced by senescence. In addition, visualization in 2D graph on a plane with Cartesian coordinates PC1 and PC2 gave distinct picture of relations within parameters and/or among wheat varieties (Kalaji et al. 2014, 2018a,c; Španić et al. 2017, Pavlović et al. 2019, Rusinowski et al. 2019).

PCA analysis elucidated a moment of senescence onset at 21 DAF in Olimpija which was confirmed by high correlation of variety Olimpija and parameters describing dissipation process ($q_{00}$ and DL/R/RC). Furthermore, at 28 DAF, the same parameters ($q_{00}$ and DL/R/RC) grouped with Chl a/b, ABS/RC, and TR/RC indicating progressive photooinhibition of PSII in the flag leaves of Olimpija which is in accordance with flattened fluorescence intensity OJIP curve. Similar results were obtained by Kalaji et al. (2018a) in drought-stressed Tilia cordata plants. On contrary, variety Alka correlated with Chl contents, performance indexes, and parameters describing electron transport.

As photosynthesis is of a great importance during wheat grain-filling period and directly influences grain yield, delayed flag leaf senescence is desirable trait in wheat breeding programs. Zhang et al. (2006) found correlation between delayed flag leaf senescence and higher grain yield of wheat. Summarizing the results presented in current study, alterations in flag leaf photochemistry strongly suggested that variety Alka managed to delay flag leaf senescence onset whereas modifications of photosynthetic apparatus and thus photosynthetic efficiency decline started earlier in Žitarka and particularly Olimpija. This coincided with the grain yield potential of those three varieties, whereas according to catalogues yearly published by Agricultural Institute Osijek (www.poljinos.hr), Alka was the most yielding variety with genetic potential of 11 t ha$^{-1}$, followed by Žitarka and Olimpija with 9 and 8 t ha$^{-1}$, respectively. We confirmed hypothesis that ‘stay-green’ trait enabled Alka to retain green leaves longer after anthesis, thus improving grain yield potential, compared to Žitarka and notably Olimpija with lower grain yield potential. Alka showed that duration of photosynthetic processes in wheat flag leaves influence the grain yield significantly, which was previously found by many authors (Zhang et al. 2006, Yang et al. 2007, Gregersen et al. 2013, Thomas and Ougham 2014, Viljevac Vuletić et al. 2019).

Based on presented data, although investigated wheat varieties have similar maturation time, senescence onset was triggered earlier in the flag leaves of Olimpija, compared to Žitarka and Alka. OJIP curves, with L- and K-bands presented, implied conclusions that flag leaves of variety Alka detained the longest photosynthetic efficiency, while the first symptoms of senescence onset were visible through positive L- and K-bands in the flag leaves of variety Olimpija. Furthermore, a shift in I–P phase in the wheat flag leaves suggested a reduction of the first acceptors in PSI, implying a degradation of photosynthetic apparatus beside appearance of photoinhibition. $P_{\text{total}}$ proved to be a much more sensitive indicator of senescence than $P_{\text{sys}}$. Parameters $q_{00}$ and DL/R/RC highly correlated with variety Olimpija suggesting photoinhibition onset followed by disassembly of photosynthetic apparatus. The flag leaves of Olimpija could not maintain their photosynthetic activity for a longer period of time, experiencing very early leaf senescence. A consequence of that is the lower genetic potential for grain yield, compared to Žitarka and particularly Alka. Along with $P_{\text{total}}$, principal component analysis elucidated $q_{00}$ and DL/R/RC as early indicators of senescence in the wheat flag leaves and thus showed that multivariate approach may be useful in the senescence onset detection. Multivariate approach for analysing the data obtained by ChlF can be useful tool for wheat breeders allowing them to enhance breeding selection from physiological point of view to obtain lines with ‘stay-green’ trait thus improving productivity.

References

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PHOTOSYNTHETIC PERFORMANCE DURING LEAF SENESCENCE IN WHEAT


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