Original scientific paper
DOI: /10.5513/JCEA01/23.2.3437
Journal of Central European Agriculture, 2022, 23(2), p.305-317

Chlorophyll *a* fluorescence as tool in breeding drought stress-tolerant soybean

Fluorescenca klorofila *a* kao alat u oplemenjivanju soje tolerantne na sušu

Maja MATOŠA KOČAR[1] (✉), Ana JOSIPOVIĆ[2], Aleksandra SUDARIĆ[1,3], Tomislav DUVNJAK[1], Marija VILJEVAC VULETIĆ[1], Monika MARKOVIĆ[4], Antonela MARKULJ KULUNDŽIĆ[1]

[1] Agricultural Institute Osijek, Južno predgrađe 17, 31103 Osijek, Croatia

[2] Independent researcher; Vukovar, Croatia

[3] Center of Excellence for Biodiversity and Molecular Plant Breeding, University of Zagreb, Faculty of Agriculture, Svetošimunska cesta 25, 10000 Zagreb, Croatia

[4] Josip Juraj Strossmayer University of Osijek, Faculty of Agrobiotechnical Sciences Osijek, Vladimira Preloga 1, 31000 Osijek, Croatia

✉ Corresponding author: maja.matosa@poljinos.hr

Received: October 11, 2021; accepted: February 15, 2022

ABSTRACT

Mitigating the effects of changing climate with adaptable cultivars while reducing the input burden of additional selection criteria is becoming a priority, especially as water shortage is limiting soybean production in Europe. To evaluate the functionality of the photosynthetic apparatus in drought, chlorophyll *a* (Chl *a*) fluorescence was measured in 16 elite soybean lines in drought and conditions with sufficient water supply in V2 (second node), R1 (beginning bloom), R4 (full pod), R5 (beginning seed) and R6 (full seed) stages. Developmental stage was a significant source of variation for all parameters, and PI_{total} was chosen as the most sensitive parameter in detecting the average drought effect. Genotypes G5, G9, and G10 had superior overall functioning of the photosynthetic apparatus in drought, while the photosynthetic apparatus of G12 and G16 was the least functional. The drought effect was determined to be the most relevant in R1, R4 and R6. G14 had highest PI_{total} in drought conditions in R1 and R4, while G9 had the highest drought-stressed PI_{total} in R6. G7 had the lowest drought-stressed PI_{total} in R1, G4 and G6 had the lowest in R4, and G8 had the lowest in R6. PI_{total} proved useful in breeding for abiotic stress tolerance, especially for excluding the material with the poorest photosynthetic apparatus function, which increases the efficiency of the selection process when a large number of genotypes needs to be screened. However, genotypes with superior photosynthetic apparatus functioning should be further tested in yield trials to confirm their drought tolerance and value for use in drought conditions.

Keywords: drought susceptibility, photosynthesis, photosynthetic efficiency, stages of development

SAŽETAK

Ublažavanje negativnih posljedica klimatskih promjena stvaranjem adaptabilnih sorti bez opterećenja selekcije dodatnim troškovima od velikog je značaja, osobito jer suša sve više ograničava proizvodnju soje u Europi. Za procjenu funkcionalnosti fotosintetskog aparata u suši, fluorescencija klorofila *a* (Chl *a*) mjerena je na 16 elitnih linija soje u suši i uvjetima dovoljne opskrbe vodom u V2 (drugi nodij), R1 (početak cvatnje), R4 (puni razvoj mahuna), R5 (začetak formiranja sjemena) i R6 (puni razvoj sjemena) fazama. Razvojna faza bila je značajan izvor varijacija za sve parametre, a PI_{total} parametar je odabran kao najosjetljiviji u otkrivanju prosječnog učinka suše. Genotipovi G5, G9 i G10 imali su superiorno funkcioniranje fotosintetskog aparata u suši, a G12 i G16 najmanje funkcionalan fotosintetski aparat u suši. Utvrđeno je da je učinak suše najrelevantniji u R1, R4 i R6 fazama razvoja. Fotosintetski aparat G14 genotipa bio je najmanje osjetljiv

na sušu u R1 i R4 fazama razvoja, dok je kod G9 bio najmanje osjetljiv u R6. G7 je imao najmanje funkcionalan fotosintetski aparat u uvjetima suše u R1, G4 i G6 su imali najmanje funkcionalan fotosintetski aparata u uvjetima suše u R4, a G8 u R6. Uporaba PI_{total} u oplemenjivanju na tolerantnost prema abiotskom stresu pokazala se korisnom, posebice za isključenje materijala s najlošijim funkcioniranjem fotosintetskog aparata, što povećava učinkovitost selekcijskog procesa kada je potrebno procijeniti veliki broj genotipova. Međutim, genotipove sa superiornim funkcioniranjem fotosintetskog aparata potrebno je dodatno ispitati kako bi se potvrdila njihova tolerantnost i uporabna vrijednost u uvjetima suše.

Ključne riječi: osjetljivost na sušu, fotosinteza, fotosintetska učinkovitost, faze razvoja

INTRODUCTION

Nowadays, the frequency and severity of climate and weather extremes are increasing with drought and heat waves negatively affecting agriculture. On a global level, climate aberrations are estimated to cause a third of crop yield variability (Ray et al., 2015). Although European regions are already facing more frequent, severe, and longer-lasting droughts, if global warming would cause an increase of average global temperature by 3 °C, droughts would happen twice as often, and the absolute annual drought losses in Europe would increase to EUR 40 billion/year, with the most severe impacts in the Mediterranean and Atlantic Regions (European Commission, 2021). As soybean is mostly produced in the rainfed agricultural systems in Europe (FAO, 2021), water shortage can seriously limit production. One way to ensure yield stability in unstable and extreme weather conditions is to breed new, adaptable crop varieties that can meet the high productivity criteria.

In the early development, soybean can withstand shorter length drought without the significant yield decrease, but water needs increase from the beginning of flowering (R1), through the pod development (R3), up until the full seed development (R6), when 60 to 90% of the total crop water needs are required (Vratarić and Sudarić, 2008, Board and Kahlon, 2011). This coincides with the period from early July to late August, i.e. the summer months when high temperatures and water shortages occur regularly. As abiotic stress is the subject of many studies, efficient and reliable tools and methods for determining it are crucial. The chlorophyll *a* (Chl *a*) fluorescence measurement, being relatively fast, simple and non-invasive, is the most common method for describing leaf photosynthesis in natural conditions, and many authors have used it to prove stress in plants (Markulj Kulundžić et al., 2016; Jumrani et al., 2017; Kovačević et al., 2017; Umar and Siddiqui 2018; Killi et al., 2020; Markulj Kulundžić et al., 2021). It provides information on the photosynthetic apparatus, the electron transport chain, and the efficiency of the PSII (Strasser et al., 1995). Furthermore, Chl *a* fluorescence is used for predicting, monitoring and identifying plants' response to environmental stressors and determining their ability to adapt. It gives information on the extent of photosynthetic damage and can be used as a method of detecting drought-tolerant genotypes (Strasser et al., 2004; Kalaji et al., 2016).

In this research, the aims were: i) to investigate if drought had affected the functioning of the photosynthetic apparatus described by 23 Chl *a* fluorescence parameters in 16 tested elite soybean lines; ii) to investigate if the functioning of the photosynthetic apparatus varied depending on the timing of the drought; iii) to determine Chl *a* fluorescence parameters most indicative of drought stress in a given set of genotypes; iv) to determine genotype variability evaluated by the Chl *a* fluorescence parameter determined to be the most sensitive in drought conditions; v) to determine the developmental stages were drought stress significantly affected photosynthetic processes described by the chosen parameter and vi) to evaluate the functioning of genotypes' photosynthetic apparatus in drought conditions in those stages of development. This would enable the detection and elimination of drought-susceptible genotypes and the selection of potentially more drought-tolerant genotypes to be further tested and eventually used as parental components in crossings aiming to produce progeny less susceptible to water scarcity. In breeding programmes

that include a large number of genotypes, using easy, relatively fast and reliable methods such as measuring the Chl *a* fluorescence can reduce the costs of screening and inputs as well as facilitate the decision making process. This is especially important in the conventional, GMO-free breeding programmes which are more time-consuming.

MATERIALS AND METHODS

The research was conducted in plant pots with 16 0–I maturity group (MG) elite soybean lines previously not selected for drought stress tolerance, all created and in the property of Agricultural Institute Osijek (AIO, Osijek, Croatia). The 12 000 cm^3 of soil for each pot (22.5 cm in height, 28.5 cm in diameter) was taken from the arable soil layer (30 cm depth), sifted to eliminate plant remains and large soil aggregates. The soil is classified as anthropogenic eutric cambisol (WRB), silty clay loamy texture with following physical properties in upper soil layer: 64.7% silt, 32.5% clay, 2.8% sand, 44.8% porosity (P), 36.6% available water holding capacity (AWC), 5.2% air capacity (AC), 39.52% saturation (SAT), 23.7% permanent wilting point (PWP), and 2.75 g/cm^3 partical density (Marković et al., 2021). The AWC of the soil was determined using the gravimetric method modified by Schinner et al. (1993). Three samples per 100 g of air-dried soil were weighed, placed in an oven at 105 °C until constant weight (approximately 24 h), and afterwards weighted again. The gravimetric moisture content was calculated as the average of three soil samples, and the amount of air-dried soil for each pot was determined. Hydroscopic water in the air-dried soil was considered when calculating the watering rate so that the water content in the soil reached 100% of AWC. Since the field capacity (FC) value was known from previous laboratory analyses, the next step was to determine the watering rate needed to fill the water content to 100% AWC. For T1 (80% AWC), the watering rate was 0.0293 g/g, while for T2 (50% AWC), the watering rate was 0.0237 g/g. Each pot was filled with sieved, air dried soil, weighed and saturated with water. Approximately after a 7-hour draining of the excess water, the pots were weighed again. The amount of water was determined by weighing the pots every day to determine the amount of water consumed by the plants, i.e. the amount of water that needed to be compensated by watering. Reference pots were used so that the increasing plant biomass during the plant gowth could be taken into consideration. Water was taken from a 37 m deep well, located near the greenhouse. Water was pumped into a tank and kept near the pot trial so that the water temperature was as close as possible to the soil temperature to avoid shock. Before use, water samples were taken and analysed in the laboratory to determine the chemical and physical properties. According to the results of the analysis, the water was safe for use without restrictions (Ayers and Westcot, 1994).

The pot trials had 2 treatments and 3 repetitions per treatment and genotype. The first treatment (T1) had sufficient water supply (80% AWC), while the second one (T2) was drought stressed (50% AWC). This means that the plants were irrigated when soil water content (SWC) reached 29.3% (management allowable depletion, MAD) in T1 and 23.7% (PWP) in T2. The drought stressed treatment (T2) was simulated in 5 stages of the soybean development: the second node (V2), the beginning bloom (R1), the full pod (R4), the beginning seed (R5) and the full seed (R6) (Fehr and Caviness, 1977), on different sets of plants. Air temperature (°C) and relative air humidity (%) were measured hourly with Data logger - LOG32 (Dostmann electronic GmbH, Germany). The average monthly air temperatures (T, °C) and the average monthly relative air humidity (RH, %) during the experiment (May–August) and respective long-term averages (LTA) for Osijek, Croatia are listed in table 1.

Sowing was performed by inserting 6 seeds, 2 in each corner of the equilateral triangle with 10 cm long sides, after which 500 cm^3 of sand was added to each pot. After emergence, plants were thinned to 3 per pot. Considering there were enough P and K in the soil, only N fertiliser UREA (46% N) was added 2 times during the growing season each year. Systemic insecticide (active ingredient: thiamethoxam) was applied against the red spider mite (*Tetranychus urticae* Koch), as soon as the first symptoms were observed.

Table 1. The average monthly air temperatures (T, °C) and the average monthly relative air humidity (RH, %) measured with data logger during the experiment (May–August) and respective long-term averages (LTA, 1981–2010) for Osijek–Čepin, Croatia (Croatian Meteorological and Hydrological Service)

	Data logger		LTA (1981–2010)	
	T (°C)	RH (%)	T (°C)	RH (%)
May	16.63	74.61	17.12	69
June	27.66	57.82	20.06	70.57
July	26.71	59.30	21.95	68.47
August	25.55	52.65	21.31	70.8

Plants were grown inside the greenhouse until R1. After R1, they were taken outside and placed under a polyethylene foil roof to prevent rain from watering the soil in pots. The Chl *a* fluorescence was measured in both treatments in cloudless conditions when SWC reached PWP in T2 (T1 was maintained at MAD at all times), in each of the 5 stages of the soybean development (V2, R1, R4, R5, R6). After Chl *a* fluorescence was measured, the T2 soil was irrigated, and SWC was increased and maintained at 80% AWC (MAD) until harvest for both treatments.

The Chl *a* fluorescence was determined on 3 plants per repetition by the saturation pulse method (Kalaji et al., 2014) on a middle leaflet of the last fully developed trifoliate with the Handy Plant Efficiency Analyzer (PEA, Hansatech Instruments, King's Lynn, Norfolk, UK). The measurements were taken between 7:00 and 9:00 AM. The leaves were adapted to dark with the light exclusion clips for a minimum of 30 minutes, after which the Chl *a* fluorescence transients were induced using a pulse of saturating red light (peak at 650 nm, 3200 µmol/m^2s^1). Data recorded by measuring Chl *a* fluorescence, expressed in relative units, were used for calculating the parameters according to Strasser et al. (2004) (Table 2).

The data set consisted of 1440 inputs for each recorded and calculated Chl *a* fluorescence parameter (five developmental stages, 16 genotypes, 2 treatments, 3 repetitions, 3 measurements per repetition). Chl *a* fluorescence parameter calculations were performed in Microsoft Excel according to Strasser et al. (2004) and Yusuf et al. (2010) from the recorded data. The significant sources of variation (developmental stage, genotype, treatment, all interactions) for all Chl *a* fluorescence parameters were determined with the three-way analysis of variance (ANOVA). The relationships between parameters for which all sources of variation proved significant were evaluated based on Pearson's correlation coefficients to determine which of them could be excluded from further analyses without losing information necessary for decision making. Both analyses were performed with Statistica 12.0 software (StatSoft Inc., 2013).

The strength of the correlation was determined based on the scale reported by Evans (1996). The difference between the treatments for chosen parameters was evaluated with the Bonferroni post-hoc test, which corrects the false positives possibly occurring in multiple comparisons. For determining genotype variability in drought-stressed conditions (T2), the parameter with the largest relative difference between treatments (determined with the Bonferroni test) was chosen. The two-way ANOVA was applied for determining the significance of the sources of variation (stage of development, genotype and their interaction) in drought-stressed conditions, and Fisher's least significant difference test (LSD test) at $P<0.05$ level was used for determining the differences between genotypes in parameter values. A radar plot visualising the difference between T1 and T2 parameter values was constructed in Microsoft Excel. Parameter values were analysed

Table 2. The chlorophyll *a* fluorescence parameters

The recorded Chl *a* fluorescence data	
F_0	Fluorescence intensity at 50 μs – step O; minimum fluorescence
F_{300}	Fluorescence intensity at 300 μs
F_J	Fluorescence intensity at 2 ms – step J
F_I	Fluorescence intensity at 30 ms – step I
F_m	Maximum fluorescence – step P
The fluorescence parameters calculated from the recorded data according to Strasser et al. (2004) and Yusuf et al. (2010)	
$F_v = F_m - F_0$	Maximum variable Chl fluorescence
$V_J = (F_J - F_0)/(F_m - F_0)$	Variable fluorescence at step J
$V_I = (F_I - F_0)/(F_m - F_0)$	Variable fluorescence at step I
$ABS/RC = M_0x(1/V_J)x1/ TR_0/ABS$	Absorption flux, effective antenna size of an active reaction centre (RC)
$TR_0/RC = M_0x(1/V_J)$	Trapped energy flux leading to reduction of plastoquinone (Q_A) per active RC
$ET_0/RC = M_0x(1/V_J) x (1 - V_J)$	Electron transport flux per active RC
$DI_0/RC = (ABS/RC) - (TR_0/RC)$	Dissipation flux per active RC
$RE_0/RC = M_0x(1/V_J) x (1 - V_I)$	Electron flux leading to the reduction of the PSI end acceptor per active RC
$RC/CS_0 = TR_0/ABSx(V_J/M_0)xABS/CS_0$	Density of active PSII RCs per cross-section
$TR_0/ABS = 1 - (F_0/F_m)$	The maximum quantum yield of PSII photochemistry
$ET_0/ABS = TR_0/ABS x (1 - V_J)$	The quantum yield of electron transport
$RE_0/ABS = TR_0/ABS/(1 - V_I)$	Quantum yield of electron transport from Q_A- to final PSI acceptors
$RE_0/ET_0 = (1 - V_I)/(1 - V_J)$	Probability that an electron from the intersystem electron carriers is transported to the PSI end acceptor
$RC/ABS = M_0x(1/V_J) x (1/TR_0/ABS)$	Density of active reaction centers on Chl *a* basis
$TR_0/DI_0 = F_v/F_0$	Ratio of the flow of captured photons and energy dissipation
$ET_0/(TR_0 - ET_0) = (F_m - F_J)/(F_J - F_0)$	Electron transport further than primary acceptor Q_A-
$PI_{ABS} = RC/ABSxTR_0/DI_0xET_0/(TR_0 - ET_0)$	Performance index on absorption basis, efficiency of energy conservation from absorbed photons to reduction of intersystem electron carriers
$PI_{total} = PI_{ABS}xRE_0/ET_0/(1 - RE_0/ET_0)$	Performance index for energy conservation from exciton to the reduction of PSI end acceptors

with the two-way ANOVA (genotype, treatment and their interaction as sources of variation) in each of the developmental stages separately to determine if the timing of the drought was significant. To determine the difference between genotypes in developmental stages where treatment was a significant source of variation, parameter values from drought-stressed plants were subjected to Fisher's LSD test (P<0.05).

RESULTS AND DISCUSSION

The research conducted in pots with 16 soybean genotypes (G1 – 16) in sufficient water supply (T1) and drought-stressed (T2) conditions in five stages of soybean development (V2, R1, R4, R5, R6) included 23 Chl *a* fluorescence parameters. The three-way ANOVA indicated significant (P<0.05) differences between average values from different stages of development for all tested parameters (data not shown). This is expected because the photosynthetic apparatus functioning, described by Chl *a* fluorescence parameters, is associated with chlorophyll content in leaves, which is well known to change during leaf development (Balazadeh et al., 2008; Lo Piccolo et al., 2018; Sitko et al., 2019). Average values per treatment significantly (P<0.05) differed for all parameters except F_v, V_J, DI_0/RC, ET_0/ABS, RC/ABS, ET_0/(TR_0 - ET_0) and PI_{ABS} (data not shown). Although these parameters describe photosynthetic processes prone to stress effect (Strasser et al., 2004; Kalaji et al., 2016), the values tested here are averages from five developmental stages, which could mean that their changes per developmental stage were either not large enough for the average change to be significant, or they did not change at all in some stages, decreasing the overall average difference. As the aim of this research was to use only the most sensitive parameters, these were excluded. Average values per genotype significantly (P<0.05) differed for all parameters except DI_0/RC and PI_{ABS} (data not shown). The fact that most parameters determined genotype variability indicates there is room for the selection of the improved photosynthetic performance among the given material, even though all genotypes are from the same breeding programme and of the same MG.

All sources of variation, including interactions, proved significant only for V_I, F_0/F_m, TR_0/ABS, ABS/RC RC/CS_0, TR_0/DI_0, PI_{total} (Table 3), so these parameters were considered sensitive enough for evaluating the differences between genotypes in photosynthetic apparatus functioning when affected by drought stress. As some of those parameters are mathematically connected (Strasseer et al. 2004), the correlation analysis was used to quantify those relations and determine which of them could be excluded without losing information necessary

Table 3. Mean squares and degrees of freedom (df) for different sources of variation from the three-way ANOVA for chosen chlorophyll *a* fluorescence parameters (V_I, F_0/F_m, TR_0/ABS, ABS/RC, RC/CS_0, TR_0/DI_0, PI_{total}) tested in 16 soybean genotypes (G1–16) in two treatments (T1 and T2) across 5 developmental stages (V2, R1, R4, R5, R6). Descriptions of used chlorophyll *a* fluorescence parameters are in table 2

Source of variation	Mean squares							
	df	V_I	F_0/F_m	TR_0/ABS	ABS/RC	RC/CS_0	TR_0/DI_0	PI_{total}
Stage (S)	4	0.59*	0.04*	0.04*	48.85*	276773.8*	32.19*	744.22*
Treatment (T)	1	0.28*	0.002*	0.003*	1.22*	15024.49*	4.06*	126.9*
Genotype (G)	15	0.01*	0.001*	0.001*	0.12*	708.66*	0.83*	16.63*
S x T	4	0.06*	0.004*	0.004*	2.7*	13135.48*	3.06*	79.24*
S x G	60	0.01*	0.001*	0.001*	0.25*	605.27*	0.59*	20.67*
T x G	15	0.004*	0.001*	0.001*	0.17*	540.33*	0.72*	4.79*
S x T x G	60	0.003*	0.001*	0.001*	0.13*	480.01*	0.49*	6.14*

* – Significant (P<0.05)

for decision making. Parameters that could be used one instead of the other without losing data are those with very strong and strong correlations between them. A very strong positive correlation was determined between TR_0/ABS and TR_0/DI_0. A strong negative correlation was determined between ABS/RC and RC/CS_0, while a very strong negative correlation was determined between V_I and PI_{total}, F_0/F_m and TR_0/ABS, as well as between F_0/F_m and TR_0/DI_0 (Table 4). It could therefore be argued that TR_0/ABS, ABS/RC and PI_{total} would provide the same amount of data on drought susceptibility as all seven parameters together.

TR_0/ABS is one of the most frequently used Chl *a* fluorescence parameters for determining the effect of environmental stress on the photosynthetic activity of plants and evaluating their health status under stressful conditions (Kalaji et al., 2016). Although statistically significant ($P<0.05$), the average difference between TR_0/ABS in drought-stressed plants (T2 TR_0/ABS) and plants grown in sufficient water supply (T1 TR_0/ABS) was very small (0.34%; Figure 1). Furthermore, both T1 and T2 TR_0/ABS values (Figure 1) were very near the value considered optimal (0.83) for most of the plant species, according to Björkman and Demmig-Adams (1995). This may be explained by the fact that TR_0/ABS is reportedly not appropriate for determining the early drought stress symptoms in plants (Bukhov and Carpentier, 2004; Ohashi et al., 2006), which may mean that stress in this research did not last long enough to cause notable changes. Regardless of the cause, as the maximum quantum yield of PSII photochemistry was near-optimal in plants subjected to drought, this parameter was considered not appropriate for further analyses.

The functional size of the active RCs' antenna complex (ABS/RC) increased as a result of drought (Figure 1) by 2.63% on average. This increase indicates that some RCs were inactivated as a result of drought stress (Kalaji et al., 2016), which is expected. Ergo et al. (2021) noted a 34% increase in ABS/RC in two soybean genotypes five days after the drought treatment started compared to the plants grown in sufficient water supply, and a 44% increase 32 days after the drought treatment started. Markulj Kulundžić et al. (2021) noted an ABS/RC increase range of 7-33% in eight sunflower hybrids exposed to a combination of increased temperatures and high irradiation. Bano et al. (2020) reported that drought caused an 11.6% ABS/RC decrease in drought-tolerant mung bean cultivar and a 69% increase in a sensitive one. Although statistically significant, the reductions in apparent antenna size of PSII caused by drought conditions were relatively small in this research compared to other mentioned studies, so this parameter was excluded from further analyses as well.

Table 4. Pearson's correlation coefficients (r) for 8 chosen chlorophyll *a* fluorescence parameters (V_I, F_0/F_m, TR_0/ABS, ABS/RC, RC/CS_0, TR_0/DI_0, PI_{total}) tested in 16 soybean genotypes (G1–16) in 2 treatments (T1 and T2) across 5 developmental stages (V2, R1, R4, R5, R6). Descriptions of used chlorophyll *a* fluorescence parameters are in Table 2

	Pearson's correlation coefficients (r)					
	F_0/F_m	TR_0/ABS	ABS/RC	RC/CS_0	TR_0/DI_0	PI_{total}
V_I	-0.09	0.09	0.32	0.16	0.13	-0.8
F_0/F_m		-0.99	0.54	-0.45	-0.98	-0.15
TR_0/ABS			-0.54	0.45	0.98	0.15
ABS/RC				-0.64	-0.52	-0.56
RC/CS_0					0.48	0.07
TR_0/DI_0						0.07

* - Significant; ns - Non-significant ($P<0.05$)

PI_{total} detects the combined changes at each fluorescence transient functional step, including the overall efficiency of light energy absorption (RC/ABS), quantum yield of excitation energy trapping (TR_0/DI_0), probability of a trapped exciton moving an electron further along the electron transport chain than Q_A ($ET_0/(TR_0-ET_0)$) and the probability of PSI reducing its end acceptors ($RE_0/(ET_0-RE_0)$) (Strasser et al., 2004, Yusuf et al., 2010). Among the three chosen parameters, PI_{total} had the largest average difference (13.56%) between drought-stressed plants (T2 PI_{total}) and plants grown in sufficient water supply (T1 PI_{total}; Figure 1), indicating it was overall probably more sensitive to stress compared to TR_0/ABS and ABS/RC. Its higher sensitivity to the unfavourable environmental changes compared to other fluorescence parameters and close relation to plant's vitality, e.i. growth and tolerance to stress conditions, were previously reported (Oukarroum et al., 2007; Tsimilli-Michael and Strasser, 2008; Yusuf et a., 2010; Pavlović et al., 2019; Mihaljević et al., 2021).

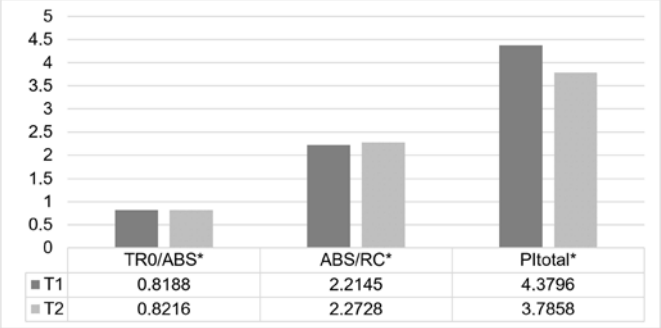

Figure 1. Average TR_0/ABS, ABS/RC and PI_{total} in plants grown in sufficient water supply (T1) and drought-stressed conditions (T2). The values are averaging data for 16 soybean genotypes (G1 – 16) in 5 developmental stages (V2, R1, R4, R5, R6) with 3 repetitions per genotype. Statistically significant differences (ANOVA, Bonferroni test $P<0.05$) between T1 and T2 parameter values are indicated by asterix (*). Descriptions of used chlorophyll *a* fluorescence parameters are in table 2

Based on the given references and presented results, PI_{total} was chosen for further analyses. As the three-way ANOVA indicated a significant effect of treatment for PI_{ABS} (Table 3), data for T2 PI_{ABS} was analysed individually using the two-way ANOVA, with developmental stage, genotype and their interaction as sources of variation. All sources of variation were significant ($P<0.05$; data not shown). The Fisher's LSD test ($P<0.05$) was used for evaluating differences between genotypes in average functioning of their photosynthetic apparatus in drought stress (T2 PI_{ABS}, Figure 2). G12 and G16 had the smallest T2 PI_{total}, while G5, G9 and G10 had the largest (Figure 2). As PI_{total}, describing the average functioning of the photosynthetic apparatus (Strasser et al., 2004), can be representative of plant's reaction in stressful conditions and its vitality (Oukarroum et al., 2007; Tsimilli-Michael and Strasser, 2008), we could argue that G5, G9 and G10 should be favoured, while G12 and G16 should be excluded from the breeding programme aimed at increasing drought tolerance. However, as drought tolerance is a complex trait (Board and Kahlon, 2011), such decisions should not be taken lightly. Nevertheless, this criterion could be helpful in pre-selection for differentiating between larger sets of genotypes. In this way, genotypes with the poorest photosynthetic apparatus functioning in abiotic stress could be eliminated, increasing efficiency and lowering the costs of breeding.

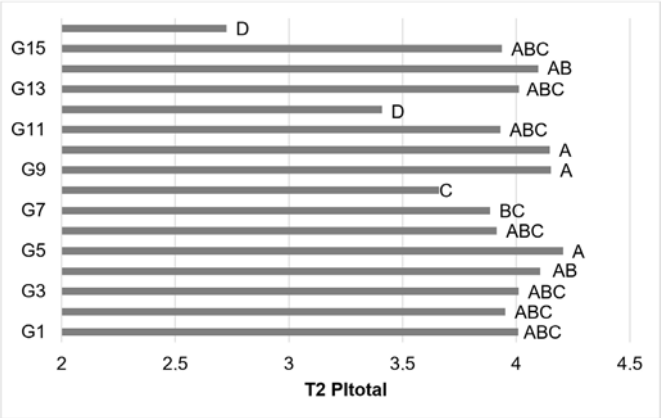

Figure 2. The average performance index for energy conservation from exciton to the reduction of PSI end acceptors in drought-stressed plants (T2 PI_{total}). Each data averages values from 5 developmental stages (V2, R1, R4, R5, R6) with 3 repetitions per genotype. Genotype T2 PI_{total} data marked with the same capital letters are not significantly different (Fisher's LSD test, $P<0.05$)

Abiotic stress usually causes a decrease in PI_{total} indicating inhibition of the PSII activity and structural and/or functional damage of the PSI, e.i. a ˝loss˝ in its ability for energy conservation (Oukarroum et al., 2007; Yusuf et al., 2010; Pavlović et al., 2019; Mihaljević et al., 2021).

The decrease was confirmed for average PI_{total} in this research as well (Figure 1). Quantification of PI_{total} change in abiotic stress could allow the selection of genotypes that have the smallest decrease, i.e. genotypes with more stable functioning of their photosynthetic apparatus. Therefore, among the genotypes with superior average functioning of the photosynthetic apparatus in drought stress, those that have increased PI_{total} or the smallest PI_{total} decrease in drought-stressed conditions compared to conditions with sufficient water supply should be favoured. To quantify the difference, the T2 PI_{total} values were expressed relative to the T1 PI_{total} (Figure 3). Most of the genotypes had expected reactions as their PI_{total} decreased in drought stress. Nevertheless, G9 and G15 had an increase in PI_{total} in drought-stressed plants, indicating a ˝gain˝ in the ability for energy conservation in stressful conditions (Yusuf et al., 2010) e.i., a superior efficiency of the photosynthetic apparatus in drought compared to other genotypes.

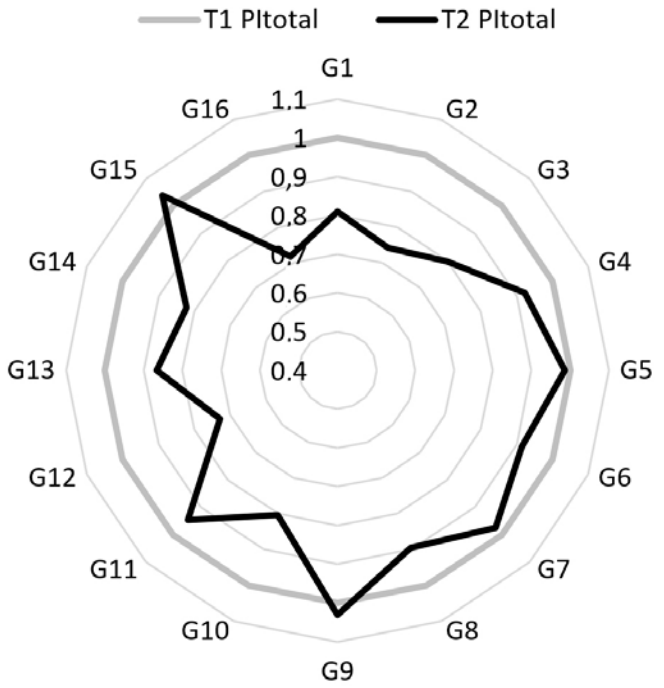

Figure 3. Radar plot of average performance index for energy conservation from exciton to the reduction of PSI end acceptors (PI_{total}) tested in 16 soybean genotypes (G1 – 16) in 2 treatments (T1 and T2). Each data averages values from 5 developmental stages (V2, R1, R4, R5, R6) with 3 repetitions per genotype. Values in plants grown in drought-stressed plants (T2 PI_{total}) were expressed relative to the PI_{total} values in plants grown in sufficient water supply (T1 PI_{total})

Considering G9 had one of the highest average T2 PI_{total}, and G15 had high T2 PI_{total} (Figure 2) as well, these two genotypes should be favoured in selection. Among the genotypes with decreased PI_{total} in drought stress, the smallest decrease was determined for G5 (1.48%; data not shown). This was one of the genotypes with the highest average T2 PI_{total} (Figure 2), so it can be argued that the functioning of its' photosynthetic apparatus is efficient and relatively stable. In G12 and G16, both having the lowest average T2 PI_{total}, the decrease was 27.21% and 28.19%, respectively (data not shown).

It is well known that the effects of drought stress depend on the timing, i.e. the developmental stage of its occurrence. To determine the sensitivity of the processes it describes to drought stress occurring at different times during the soybean vegetation period, the differences between T1 PI_{total} and T2 PI_{total} were tested in five developmental stages (V2, R1, R4, R5, R6; Figure 4) separately, with the two-way ANOVA (genotype, treatment and their interaction as sources of variation) followed by the Fisher's LSD test (P<0.05; Figure 5a-c).

PI_{total} significantly changed when drought stress was initiated in R1, R4 and R6, with the most prominent change occurring in R4 (Figure 4).

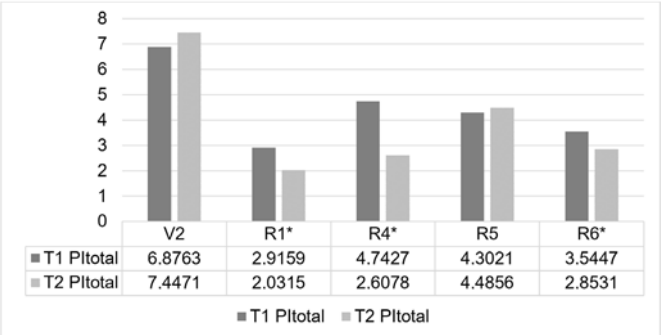

Figure 4. Average performance index for energy conservation from exciton to the reduction of PSI end acceptors (PI_{total}) in plants grown in sufficient water supply (T1 PI_{total}) and drought-stressed conditions (T2 PI_{total}) for each of the 5 developmental stages (V2, R1, R4, R5, R6). The values are averaging data for 16 soybean genotypes (G1 – 16) with 3 repetitions per genotype. Statistically significant differences (Fisher's LSD test; P<0.05) between T1 PI_{total} and T2 PI_{total} are indicated by asterix (*)

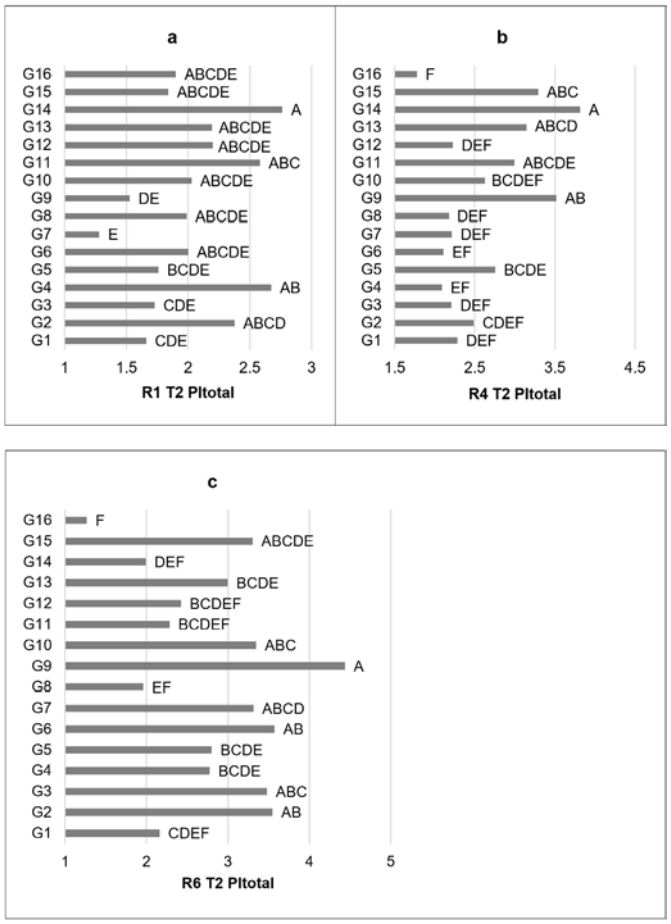

Figure 5. The average performance index for energy conservation from exciton to the reduction of PSI end acceptors in drought-stressed plants (T2 PI_{total}) in R1 (a), R4 (b) and R6 (a) developmental stage. In each developmental stage, T2 PI_{total} data marked with the same capital letters are not significantly different (Fisher's LSD test, $P<0.05$)

In the reproductive stages water stress significantly affects soybean grain yield, as needs are known to be high, resulting in an earlier onset of physiological changes in plants due to the imposed stressful conditions (Vratarić and Sudarić, 2008; Board and Kahlon, 2011; Cui et al., 2019). On the other hand, soybean plants are known to be less sensitive to water scarcity in the early development (Vratarić and Sudarić, 2008, Board and Kahlon, 2011; Cui et al., 2019), which could explain why the difference between average PI_{total} treatment values was not significant in V2. Genotype PI_{total} variability was confirmed in all developmental stages, while the genotype and treatment interaction was a significant source of variation in R1, R4 and R6 (data not shown). To determine which genotypes had superior functioning of the photosynthetic apparatus in drought-stressed conditions in developmental stages with significant treatment variation, T2 PI_{total} values from R1, R4 and R6 were subjected to one-way ANOVA followed by Fisher's LSD test ($P<0.05$; Figure 5a-c).

As it can be seen from figure 5, genotypes differed in their photosynthetic apparatus functioning in drought stress quantified by PI_{total} (T2), depending on the developmental stage. In R1 and R4, the photosynthetic apparatus of G14 was the least affected by drought (Figure 5a-b). The largest drought effect in R1 was determined in G7 (Figure 5a). Although G7 had good overall photosynthetic apparatus functioning in drought (Figure 2), its performance in R1 and R4 was not as good, but it improved in R6 (Figure 5a-c). It indicated it was more tolerant to drought during seed filling than in earlier reproductive stages. The same was true for G9, which had the most functional drought-stressed photosynthetic apparatus in R6 (Figure 5c). G9 had one of the best rankings for T2 PI_{total} averaging all five developmental stages (Figure 2), it ranked at the top in R4 (Figure 5b), but had among the lowest T2 PI_{total} in R1 (Figure 5a), indicating its good stress tolerance during the intensive pod development and seed filling, but not at the beginning of flowering. In R4 and R6, the lowest T2 PI_{total} was calculated for G16 (Figure 5b-c). G16 is expected to show the most susceptibility to drought stress, as its T2 PI_{total} averaging all five stages of development was among the lowest (Figure 2). Among the genotypes previously not excluded from the selection process because of their poor overall photosynthetic apparatus functioning, G4 and G6 had the lowest T2 PI_{total} in R4 (Figure 5b), indicating their susceptibility to drought occurring at the time pods are fully formed. In R6, among the genotypes with good overall photosynthetic apparatus functioning (Figure 2), G8 had the lowest T2 PI_{total} (Figure 5c). It had almost the same ranking in R4 (Figure 5b) and somewhat better in R1 (Figure 5a), meaning it was the least susceptible to drought at the beginning of flowering. The other two genotypes with the highest T2 PI_{total} averaging all five developmental stages (G5 and G10, Figure 2) ranked

relatively well in R1, R4 and R6 (Figure 5a-c), indicating a more stable reaction to stress. Differences in genotype reaction based on drought timing confirm the need to determine when drought is most likely to occur in the area for which the genotypes are being created and evaluate the reaction to drought stress in that particular stage of development.

CONCLUSION

In 16 tested elite soybean lines, drought affected the functioning of the photosynthetic apparatus. The timing of the drought was a significant source of variation. PI_{total} was chosen as the most sensitive in detecting the average drought stress effect in tested genotypes across five developmental stages (V2, R1, R4, R5 and R6). According to PI_{total} in drought stress averaging all stages of development, G5, G9, and G10 stood out for having superior functioning of the photosynthetic apparatus. G5 had a relatively stable functioning irrelevant of water availability, while G9 and G15 had improved functioning in drought stress. On the other hand, G12 and G16 could be excluded from the selection process as their photosynthetic apparatus was less functional in drought compared to other genotypes.

Drought significantly affected overall photosynthetic apparatus functioning at the beginning of flowering (R1), during pod development (R4) and during seed filling (R6). Among genotypes with good overall photosynthetic apparatus functioning, G14 was the least susceptible to drought occurring at the beginning of flowering (R1) and during pod development (R4), while G9 was the least susceptible to drought occurring during seed filling (R6). At the beginning of flowering (R1), G7 had the poorest functioning of the photosynthetic apparatus in drought conditions, G4 and G6 had the poorest functioning of the photosynthetic apparatus in drought conditions during pod development (R4), and G8 had the least functional photoisynthetic apparatus during seed filling (R6). These genotypes could be excluded from the selection aiming to decrease drought stress susceptibility as well. PI_{total} proved useful for evaluating stress effect and excluding the material with the poorest photosynthetic apparatus functioning in stressful conditions, which is particularly important when large numbers of genotypes need to be screened. However, genotypes with superior photosynthetic apparatus functioning should be further tested in yield trials to confirm their drought tolerance and value for use in drought conditions.

REFERENCES

Adams, W. W. III., Demmig-Adams, B. (2004) Chlorophyll fluorescence as a tool to monitor plant response to the environment. In: Papageorgiou, G.C., Govindjee, G., eds. Chlorophyll fluorescence: A signature of photosynthesis. Advances in photosynthesis and respiration series. Dordrecht: Springer, 583-604. DOI: https://doi.org/10.1007/s11738-016-2113-y

Ayers, R. S., Westcot, D. W. (1994) Water quality for agriculture. FAO Irrigation and Drainage Paper 29, Revision 1. Rome: FAO, 1-174.

Balazadeh, S., Parlitz, S., Mueller-Roeber, B., Meyer, R.C. (2008) Natural developmental variations in leaf and plant senescence in Arabidopsis thaliana. Plant Biology, 10, 136-147. DOI: https://doi.org/10.1111/j.1438-8677.2008.00108.x

Bano, H., Athar, H., Zafar, Z. U., Kalaji, H. M., Ashraf, M. (2020) Linking changes in chlorophyll *a* fluorescence with drought stress susceptibility in mung bean [Vigna radiata (L.) Wilczek]. Physiologia Plantarum, 172, 1244-1254. DOI: https://doi.org/10.1111/ppl.13327

Björkman, O., Demmig-Adams, B. (1995) Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze, E.D., Caldwell, M.M., eds. Ecophysiology of photosynthesis. Berlin: Springer, 17-47. DOI: https://doi.org/10.1007/978-3-642-79354-7

Board, J. E., Kahlon, C. S. (2011) Soybean yield formation: What controls it and how it can be improved. In: El-Shemy, H. A. eds. Soybean Physiology and Biochemistry. InTech, 1-36. DOI: http://doi.org/10.5772/17596

Bukhov, N. G., Carpentier, R. (2004) Effects of water stress on the photosynthetic efficiency of plants. In: Papageorgiou, G.C., Govindjee, G., eds. Chlorophyll fluorescence: A signature of photosynthesis. Advances in photosynthesis and respiration series. Dordrecht: Springer, 321-362. DOI: https://doi.org/10.1007/978-1-4020-3218-9_24

Cui, Y., Jiang, S., Jin, J., Ning, S., Feng, P. (2019) Quantitative assessment of soybean drought loss sensitivity at different growth stages based on S-shaped damage curve. Agricultural Water Menagement, 213, 821–832. DOI: https://doi.org/10.1016/j.agwat.2018.11.020

Ergo, V. V., Veas, R. E.,, Vega, C. R. C, Lascano, R., Carrera, C. S. (2021) Leaf photosynthesis and senescence in heated and droughted field-grown soybean with contrasting seed protein concentration. Plant Phisiology and Biochemistry, 166, 437 – 447. DOI: https://doi.org/10.1016/j.plaphy.2021.06.008

European Commission (2021) Communication from the Commission to the European parliament, the Council, the European economic and social committee and the committee of the regions: Forging a climate-resilient Europe - the new EU strategy on adaptation to climate change. COM(2021) 82 final, Brussels. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A52012DC0673 [Accessed 27 September 2021].

Evans, J. D. (1996) Straightforward statistics for the behavioral sciences. Pacific Grove, CA: Brooks/Cole Publishing.

FAO (2021) AQUASTAT Core Database. Food and Agriculture Organization of the United Nations. Available at: https://www.fao.org/aquastat/statistics/query/index.html [Accessed 4 October 2021].

Fehr, W. R., Caviness, C. E. (1977) Stages of soybean development. Special report No. 87. Available at: https://lib.dr.iastate.edu/specialreports/87 [Accessed 27. September 2021].

Jumrani, K., Bhatia, V. S., Pandey, G. P. (2017) Impact of elevated temperatures on specific leaf weight, stomatal density, photosynthesis and chlorophyll fluorescence in soybean. Photosynthesis Research, 131, 333-350. DOI: https://doi.org/10.1007/s11120-016-0326-y

Kalaji, H.M., Jajoo, A., Oukarroum, A., Brestic, M., Zivcak, M., Samborska, I. A., Cetner, M. D., Łukasik, I., Goltsev, V., Ladle, R. J. (2016) Chlorophyll *a* fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. Acta Physiologiae Plantarum, 38, 1-11. DOI: https://doi.org/10.1007/s11738-016-2113-y

Kalaji, H. M., Schansker, G., Ladle, R. J., Goltsev, V., Bosa, K., Allakhverdiev, S. I., Brestic, M., Bussotti, F., Calatayud, A., Dabrowski, P., Elsheery, N. I., Ferroni, L., Guidi, L., Hogewoning, S. W., Jajoo, A., Misra, A. N., Nebauer, S. G., Pancaldi, S., Penella, C., Poli, D. B., Pollastrini, M., Romanowska-Duda, Z. B., Rutkowska, B., Serodio, J., Suresh, K., Szulc, W., Tambussi, E., Yanniccari, M., Zivcak, M. (2014) Frequently asked questions about in vivo chlorophyll fluorescence: Practical issues. Photosynthesis Research, 122, 121-158. DOI: https://doi.org/10.1007/s11120-014-0024-6

Killi, D., Raschi, A., Bussotti, F. (2020) Lipid peroxidation and chlorophyll fluorescence of photosystem II performance during drought and heat stress is associated with the antioxidant capacities of C3 sunflower and C4 maize varieties. International Journal of Molecular Sciences, 21, 4846. DOI: https://doi.org/10.3390/ijms21144846

Kovačević, J., Mazur, M., Drezner, G., Lalić, A., Sudarić, A., Dvojković, K., Viljevac Vuletić, M., Josipović, M., Josipović, A., Markulj-Kulundžić, A., Lepeduš, H. (2017) Photosynthetic efficiency parameters as indicators of agronomic traits of winter wheat cultivars in different soil water conditions. Genetika, 49 (3), 891-910. DOI: https://doi.org/10.2298/GENSR1703891K

Lo Piccolo, E., Landi, M., Pellegrini, E., Agati, G., Giordano, C., Giordani, T., Lorenzini, G., Malorgio, F., Massai, R., Nali, C., Rallo, G., Remorini, D., Vernieri, P., Guidi, L. (2018) Multiple consequences induced by epidermally-located anthocyanins in young, mature and senescent leaves of Prunus. Frontiers in Plant Science, 9, 917. DOI: http://doi.org/10.3389/fpls.2018.00917

Marković, M., Krizmanić, G., Brkić, A., Atilgan, A., Japundžić-Palenkić, B., Petrović, D., Barač, Ž. (2021) Sustainable management of water resources in supplementary irrigation management. Applied science, 11, 2451. DOI: https://doi.org/10.3390/app11062451

Markulj Kulundžić, A., Kovačević, J., Viljevac Vuletić, M., Josipović, A., Liović, I., Mijić, A., Lepeduš, H., Matoša Kočar, M. (2016) Impact of abiotic stress on photosynthetic efficiency and leaf temperature in sunflower. Poljoprivreda, 22 (2), 17-22. DOI: https://doi.org/10.18047/poljo.22.2.3

Markulj Kulundžić, A., Viljevac Vuletić, M., Matoša Kočar, M., Mijić, A., Varga, I., Sudarić, A., Cesar, V., Lepeduš, H. (2021) The combination of increased temperatures and high irradiation causes changes in photosynthetic efficiency. Plants, 10 (10), 2076. DOI: https://doi.org/10.3390/plants10102076

Mihaljević, I., Viljevac Vuletić, M., Šimić, D., Tomaš, V., Horvat, D., Josipović, M., Zdunić, Z., Dugalić, K., Vuković, D. (2021) Comparative study of drought stress effects on traditional and modern apple cultivars. Plants, 10 (3), 561. DOI: https://doi.org/10.3390/plants10030561

Ohashi, Y., Nakayama, N., Saneoka, H., Fujita, K. (2006) Effects of drought stress on photosynthetic gas exchange, chlorophyll fluorescence and stem diameter of soybean plants. Biologia Plantarum, 50, 138-141. DOI: https://doi.org/10.1007/s10535-005-0089-3

Oukarroum, A., Madidi, S.E., Schansker, G., Strasser, R.J. (2007) Probing the responses of barley cultivars (*Hordeum vulgare* L.) by chlorophyll *a* fluorescence OLKJIP under drought stress and re-watering, Environmental and Experimental Botany, 60, 438-446. DOI: https://doi.org/10.1016/j.envexpbot.2007.01.002

Ray, D. K., Gerber, J. S., MacDonald, G. K., West, P. C. (2015) Climate variation explains a third of global crop yield variability. Nature Communication, 6, 5989. DOI: https://doi.org/10.1038/ncomms6989

Pavlović, I., Mlinarić, S., Tarkowska D, Oklestkova, J., Novak, O., Lepeduš, H., Vujčić Bok, V., Radić Brkanac, S., Strnad M., Salopek-Sondi B. (2019) Early Brassica crops responses to salinity stress: A comparative analysis between Chinese cabbage, white cabagge, and kale. Frontiers in Plant Science, 10, 450. DOI: https://doi.org/10.3389/fpls.2019.00450

Schinner, F., Öhlinger, R., Kandeler, E., Margesin, R. 1993. Bodenbiologische Arbeitsmethoden (2nd ed.). Berlin/Heidelberg: Springer.

Sitko, K., Rusinowski, S., Pogrzeba, M., Daszkowska-Golec, A., Gieroń, Ż., Kalaji, H., Małkowski, E. (2020) Development and aging of photosynthetic apparatus of Vitis vinifera L. during growing season. Photosynthetica, 58 (SI), 1-8. DOI: http://doi.org/10.32615/ps.2019.107

StatSoft Inc. (2013): STATISTICA Data analysis software system (Release 12) [Software]. Tulsa, OK: StatSoft Inc.

Strasser, R. J., Srivastava, A., Govindjee, G. (1995) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochemistry and Photobiology, 61, 32-42. DOI: https://doi.org/10.1111/j.1751-1097.1995.tb09240.x

Strasser, R. J., Srivastava, A., Tsimilli-Michael, M. (2004) Analysis of chlorophill a fluorescence transient. In: Papageorgiou, G.C., Govindjee, G., eds. Chlorophyll fluorescence: A signature of photosynthesis. Advances in photosynthesis and respiration series. Dordrecht: Springer, 321-362. DOI: https://doi.org/10.1007/978-1-4020-3218-9_12

Tsimilli-Michael M. and Strasser, R.J. (2008). *In vivo* assessment of stress impact on plants' vitality: Applications in detecting and evaluating the beneficial role of mycorrhization on host plants. In: Varma, A., ed. Mycorrhiza: State of the Art, Genetics and Molecular Biology, Eco-Function, Biotechnology, Eco-Physiology, Structure and Systematics. Berlin: Springer, 679-703.

Umar, M., Siddiqui, Z. S. (2018) Physiological performance of sunflower genotypes under combined salt and drought stress environment. Acta Botanica Croatica, 77, 36-44. DOI: https://doi.org/10.2478/botcro-2018-0002

Vratarić, M., Sudarić, A. (2008) Soybean. Osijek: Poljoprivredni institut Osijek.

Yusuf, M. A., Kumar, D., Rajwanshi, R., Strasser, R. J., Tsimilli-Michael, M., Govindjee, S., Sarin, N. B. (2010) Overexpression of g-tocopherol methyl transferase gene in transgenic *Brassica juncea* plants alleviates abiotic stress: Physiological and chlorophyll *a* fluorescence measurements. Biochimica et Biophysica Acta 1797, 1428–1438. DOI: https://doi.org/10.1016/j.bbabio.2010.02.002