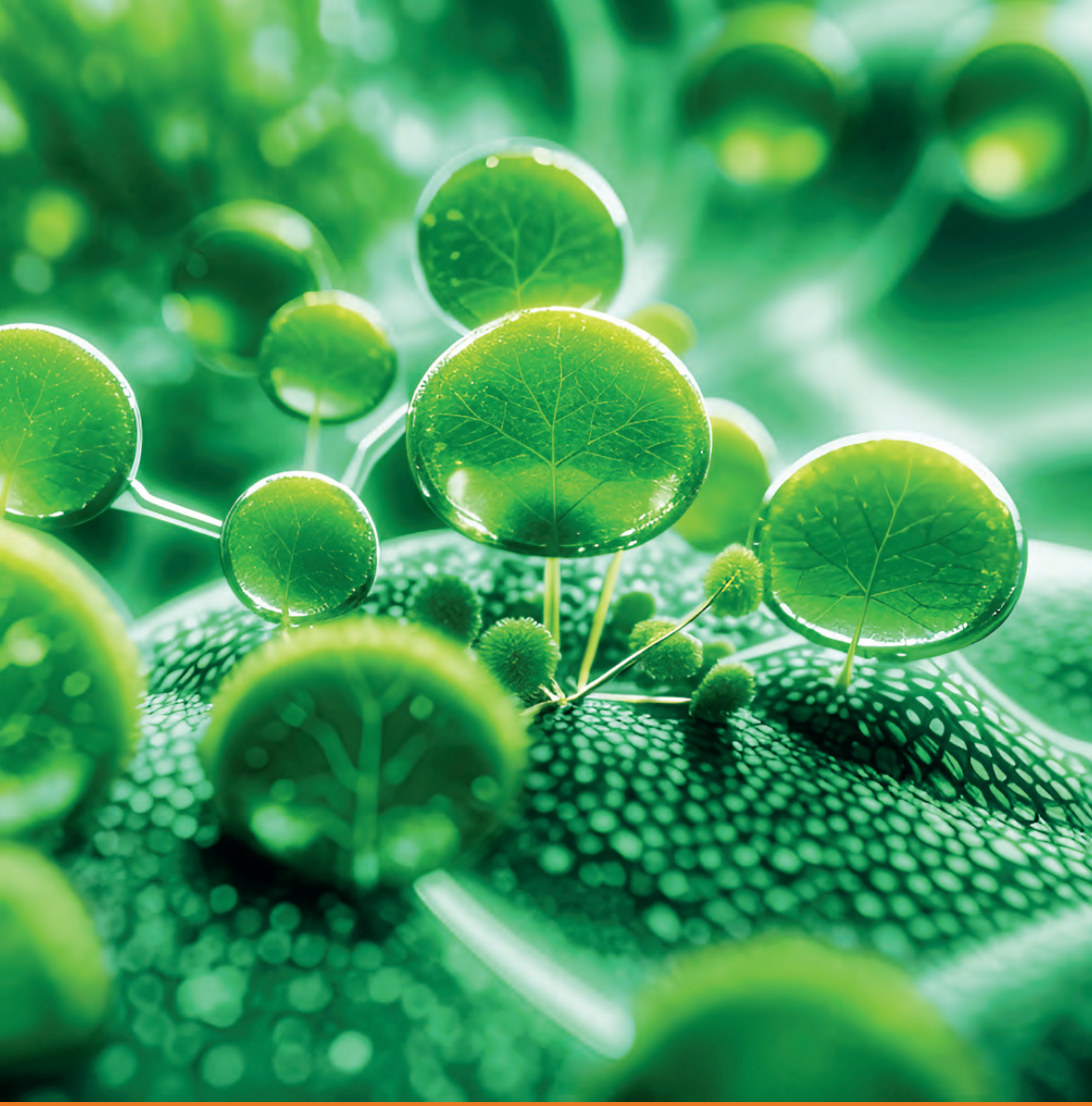

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· OSIJEK ·

CHLOROPHYLL *a* FLUORESCENCE MEASUREMENTS IN CROATIA

• FIRST TWENTY YEARS

EDITORS • Hrvoje Lepeduš • Marija Viljevac Vuletić • Zvonimir Zdunić

Chlorophyll *a* Fluorescence Measurements in Croatia - First Twenty Years

Edited by

Hrvoje Lepeduš
Marija Viljevac Vuletić
Zvonimir Zdunić

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Osijek, 2023

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Edited by

Hrvoje Lepeduš
Marija Viljevac Vuletić
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Monograph of Agricultural Institute Osijek

Contents

Part 4. Case Study

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Preface

As the monograph name itself suggests, the creation was inspired as a tribute to twenty years of the measurement of chlorophyll *a* fluorescence (ChlF) in the Republic of Croatia. Starting with basic research of the photosynthetic performance of spruce needles, research was broadened to other conifers as well as to several agricultural species with the intention to improve the breeding work at the Agricultural Institute Osijek by using the same method for screening the crops' phenotypic variability in optimal and stress conditions. The synergistic enthusiasm of biologists and agronomists led to interdisciplinary studies, which further resulted in a large number of significant research papers and projects, and ultimately, this monograph.

The monograph begins with a historical overview of the use of this method in scientific research and continues with the use of the chlorophyll fluorescence measurement method seen through two different yet very similar and closely related points of view, physiological and agronomic. Various abiotic stresses significantly affect the photosynthetic activity of plants, and through the four chapters, this monograph presents how biologists have used the ChlF in research and understanding the response of plants to the stresses they are exposed to. The next six chapters show the practical application of the ChlF method in agronomic research, mainly in elucidating the physiological mechanisms of crops under stressful environmental conditions and finding tolerant genotypes, which gives breeders a strong tailwind when creating new varieties and hybrids. The penultimate chapter presents a case study and provides a very detailed insight into the comprehensiveness and effectiveness of the ChlF method in plant material phenotyping. The last chapter discusses ChlF recent advancements in technology and analysis and the future prospects of methods that are bright, with many potential applications in agriculture, urban farming, forestry, aquatic research, climate change, and bioenergy research.

Editors

Part 1

Brief Historical Overview and Scientific Impact of Chlorophyll *a* Fluorescence Measurements in Croatia during First 20 Years

Brief Historical Overview and Scientific Impact of Chlorophyll *a* Fluorescence Measurements in Croatia during First 20 Years

Hrvoje Lepeduš

The introduction of the chlorophyll *a* measurement in Croatia was preceded by a scientific platform that was based on the investigation of spruce (*Picea abies* L. Karst.) vegetative buds and needles structure and function (*Cesar and Cesar 1988, Cesar 1989, Cesar 1992, Cesar and Bornman 1995, Cesar and Bornman 1996, Cesar et al. 1997, Lepeduš 1997, Cesar et al. 2001, Lepeduš 2001, Lepeduš et al. 2001, Lepeduš et al. 2003*). Anatomical structure, as well as structural and biochemical changes of vegetative buds and developing spruce needles, were topics studied by these investigations. As shown in **Figure 1a** and **1b**, spruce vegetative buds had light green needle primordia regardless of light deprivation due to cover made by protecting scales arranged in numerous rows (*Lepeduš 2001*). Also, certain amounts of chlorophylls and carotenoids were present in buds which corroborated with the observed weakly developed thylakoid system in their chloroplasts (*Senser et al. 1975, Lepeduš et al. 2001, Lepeduš et al. 2003*). The distribution pattern of chloroplasts in vegetative buds revealed their presence in needle primordia and medulla (**Figure 1c** and **1d**). Another interesting observation concerning the described chloroplasts was their activities during branch and needle development. They revealed an unexpected delay of photosynthetic pigments accumulation upon buds proliferation and exposure to direct sunlight (*Lepeduš et al. 2003*). To obtain the "big picture" of spruce chloroplasts functioning in different needle developmental stages, it was necessary to reach out for more advanced methods end techniques.

Since this was the post-war time in Osijek (Croatia), the lack of laboratory equipment and materials was a big obstacle for all scientists in this part of Croatia.

The only possibility for quality scientific research was to make good collaborations that would facilitate needed conditions. Previously described investigations on the spruce vegetative buds and needles photosynthetic properties were done at the Faculty of Education in Osijek (now: Faculty of Humanities and Social Sciences) as a part of projects (MZOS 122016, MZOS 0122016) headed by Prof. Vera Cesar and in collaboration with Dr. Nikola Ljubešić (then the Head of the Laboratory for Electron Microscopy at the Ruđer Bošković Institute in Zagreb, Croatia).

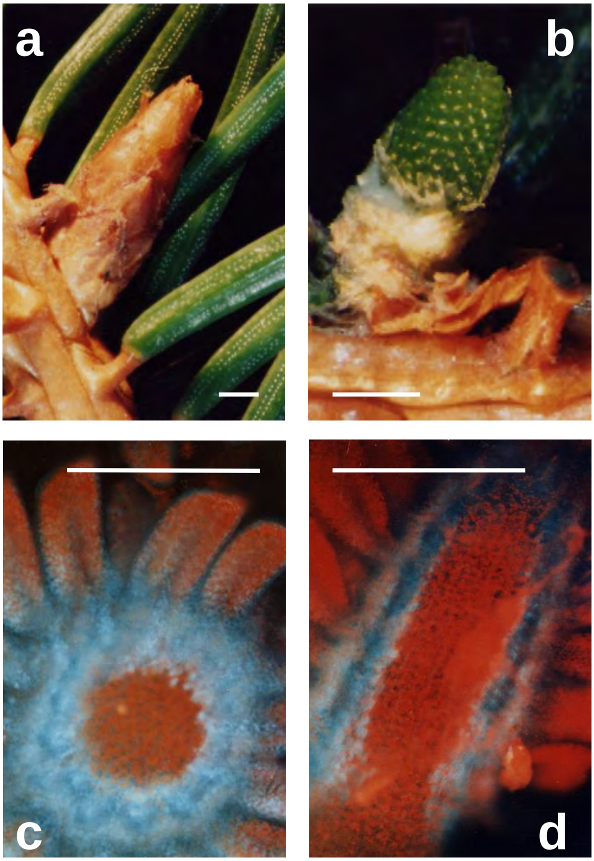

Figure 1. Vegetative bud of spruce: a – bud covered with the protective scales (cataphylls); b – bud after the cataphylls have been removed, the bright green embryonic shoot can be seen; c and d – micrographs of transversal (c) and longitudinal (d) fresh, free-hand sections of bud, agitated with violet light: red – chlorophyll, blue – cellulose in the cell walls. Chlorophyll containing plastids are present in needle primordia and medulla. The cylinder of cells around the medulla does not contain chloroplasts (source: *Lepeduš 2001*).

Further progress was made through another collaboration achieved during 2001 and 2002 with Prof. Karin Krupinska (Kiel, Germany) due to support of the Federation of European Biochemical Societies (FEBS). FEBS granted Hrvoje Lepeduš to do the experimental part of the PhD dissertation (*Lepeduš 2003*) in the Laboratory of Cell Biology (Botanical Institute in Kiel, Germany). This was a great opportunity to learn new techniques, such as chlorophyll *a* fluorescence measurement. The Laboratory of Cell Biology in Kiel was generally well equipped. Regarding photosynthetic efficiency, this included gas phase and liquid phase Clark-type oxygen electrodes (Hansatech, UK) and the "Pulse Amplitude Modulated" fluorometer (PAM100, Walz, Germany). Thereafter (in November 2002), similar equipment (the "Pulse Amplitude Modulated Photosynthesis Yield Analyzer, Mini-PAM, Walz, Germany) was purchased at the Faculty of Education in Osijek (**Figure 2**). By saturating pulse method (*Schreiber et al. 1994*), several important photosynthetic parameters were enabled to be determined *in vivo*: the maximum quantum yield of photosystem II (PSII; F_v/F_m), the effective quantum yield of PSII ($\Delta F/F'_m$), relative electron-transport rate driven by PSII (rel. ETR), the coefficient of the photochemical quenching (qP), as well as the non-photochemical quenching (NPQ) of chlorophyll *a* fluorescence.

This fitted in very nicely with other methods that were introduced parallel at the laboratory at the Faculty of Education (later on divided into several institutions and one of them was the Department of Biology in Osijek) or done in collaborations. Here, the net photosynthesis determination by oxygen evolution and dark respiration with gas phase Clark-type oxygen electrodes (Hansatech, UK), immunodetection of important photosynthetic proteins (light-harvesting complex of PSII (LHC II), cytochrome b-559 (Cyt b-559) the large subunit of Rubisco (LSU) and NADPH-protochlorophyllide oxidoreductase (POR), the translocon of the chloroplast outer envelope proteins (αToc34 and αToc159) and the translocon of the chloroplast inner envelope protein (αTic110)) by using SDS-electrophoresis and western blotting, as well as ultrastructural changes of chloroplasts done by electron microscopy (in collaboration with Dr. Nikola Ljubešić and Dr. Hrvoje Fulgosi from the Ruđer Bošković Institute in Zagreb, Croatia) must be emphasized. Combining all the mentioned methods provided the scientists in Osijek the opportunity to publish several publications in domestic and foreign scientific journals that gave further insights into molecular, structural, and functional processes that took place in conifer buds and needles (*Lepeduš and Cesar 2004, Lepeduš et al. 2005a, Lepeduš et al. 2005b, Lepeduš et al. 2005c, Fulgosi et al. 2005*).

Figure 2. The mini PAM (Walz) setup (a) and its original inventory number (b) (photo: Lepeduš H, 2006).

The next milestone in the introduction of chlorophyll *a* fluorescence measurement took place in 2007 when a Plant Efficiency Analyser (PEA, Hansatech, UK) was purchased at the Department of Biology in Osijek. This enabled the measurement of chlorophyll *a* fluorescence transients and gave the possibility to quantify a great number of different photosynthetic parameters (the JIP test, *Strasser et al. 2004*) that were compatible with data obtained by the previously mentioned saturating pulse method. Soon after, the interest in collaboration with the "Osijek group" increased among other plant orientated scientists in Osijek, mainly from Agricultural Institute Osijek, as well as in Zagreb, which resulted in further mutual research projects (**Table 1**) and quality papers, not only in conifers but in the other plant model species (*Ćurković Perica et al. 2007, Fulgosi et al. 2008, Lepeduš et al. 2008a, Lepeduš et al. 2008b, Jurić et al. 2009, Lepeduš et al. 2009, Lepeduš et al. 2010a, Lepeduš et al. 2011a, Lepeduš et al. 2011b, Brcko et al. 2012, Ful-*

gosi et al. 2012, Katanić et al. 2012, Lepeduš et al. 2012, Radić et al. 2013, Vojta et al. 2015, Begović et al. 2016, Pavlović et al. 2019a, Vojta et al. 2019, Lepeduš et al. 2020, Trstenjak et al. 2020,) as well as in the crop plants (*Lepeduš et al. 2010b, Kovačević et al. 2011, Lepeduš et al. 2012, Kovačević et al. 2013, Viljevac et al. 2013, Žuna Pfeiffer et al. 2013, Šimić et al. 2014, Antunović Dunić et al. 2015, Markulj Kulundžić et al. 2016a, Markulj Kulundžić et al. 2016b, Mlinarić et al. 2016, Kovačević et al. 2017, Lepeduš et al. 2017, Mlinarić et al. 2017a, Mlinarić et al. 2017b, Pavlović et al. 2018, Pavlović et al. 2019b, Mihaljević et al. 2020, Markulj Kulundžić et al. 2021, Mlinarić et al. 2021, Markulj Kulundžić et al. 2022a, Markulj Kulundžić et al. 2022b, Markulj Kulundžić et al. 2022c, Radić et al. 2022, Viljevac Vuletić et al. 2022*).

Thanks to the established collaborations, the education of young researchers in using chlorophyll *a* fluorescence measurements took place, too. This resulted in 14 doctoral dissertations and one M. Sc. Thesis (**Table 2**), as well as numerous graduate diploma theses. Also, the apparatuses used in the scientific research were also used in the student practicums at the Universities in Osijek, Zagreb, and Tuzla (Bosnia and Herzegovina).

In conclusion, it can be said that chlorophyll *a* fluorescence measurement had a significant impact on both fundamental and applied plant sciences development, not only in Osijek but also at institutions from other parts of Croatia. It should also be mentioned that a method was established afterwards, but independently from the "Osijek group", at some institutions in Zagreb (Faculty of Agriculture, Zagreb) and Poreč (Institute of Agriculture and Tourism Poreč), which resulted in nice publications (e.g. *Lazarević et al. 2022, Huđ et al. 2023*). The following chapters should enable more detailed insight into what was done by now, what is currently being investigated, as well as future directions of investigations in which chlorophyll *a* fluorescence will be utilized in Croatia.

Table 1. The list of the most important scientific projects that utilized chlorophyll *a* fluorescence measurements and contributed to high quality scientific papers and doctoral dissertation production in the period from 2002 until now (sources: Ministry of Science, Education and Sports od Republic of Croatia (MZOS) – Projects archive; Croatian Science Foundation (HRZZ) – Project database).

INSTITUTION	PROJECT CODE	PROJECT TITLE	PRINCIPAL RESEARCHER	NUMBER OF RESEARCHERS	THE YEAR OF THE PROJECT START
Faculty of Education Osijek (now: Faculty of Humanities and Social Sciences)	MZO 0122016	Study of spruce vegetative buds, shoots and needles	Vera Cesar	7	2002
Agricultural Institute Osijek	MZOS 073-0731674-0552	Stress physiology and agricultural characteristics of wheat and barley cultivars	Josip Kovačević	7	2007
Agricultural Institute Osijek	MZOS 073-0731674-0841	Cell and tissue differentiation in developing plant organs	Vera Cesar	15	2007
Agricultural Institute Osijek	MZOS 073-0730463-0203	Genetic analysis of mineral concentrations in maize kernels	Domagoj Šimić	5	2007
Agricultural Institute Osijek	MZOS 073-0731674-1673	Organization and function of plant cells and tissues upon the oxidative stress	Hrvoje Lepeduš	16	2007
Ruđer Bošković Institute (Zagreb)	MZOS 098-0982913-2838	Regulatory mechanisms of photosynthesis and plastid differentiation	Hrvoje Fulgosi	10	2007
Agricultural Institute Osijek	MZOS 073-1781844-1930	Biotechnological methods in fruit tree identification, selection and propagation	Zorica Jurković	7	2007
Agricultural Institute Osijek	MZOS 073-0000000-3538	Stability of sunflower genotypes on important agronomic traits and oil quality	Miroslav Krizmanić	5	2008
Agricultural Institute Osijek	IP-2013-11-5707	Genetics and physiology of tolerance to multiple stresses in maize	Domagoj Šimić	14	2014
Ruđer Bošković Institute (Zagreb)	IP-2014-09-1173	Exploring novel molecular mechanisms of alternative electron partitioning in photosynthesis	Hrvoje Fulgosi	7	2015
Ruđer Bošković Institute (Zagreb)	IP-2014-09-4359	Phytohormones in abiotic stress of Brassica crops: mechanism of tolerance and application	Branka Salopek-Sondi	14	2015
Ruđer Bošković Institute (Zagreb)	IP-2020-02-8590	Molecular mechanisms of alternative electron partitioning in photosynthesis	Hrvoje Fulgosi	5	2020

Table 2. The list of the Doctoral dissertations and Master of Science Theses that came out from the scientific projects listed in **Table 1**, in which the chlorophyll *a* fluorescence measurements were utilized.

POSTGRADUATE STUDY or INSTITUTION	PROJECT(S) CODE	DISSERTATION / M. Sc. THESIS TITLE	CANDIDATE	MENTOR(S)	THE YEAR OF DEFENCE
Faculty of Natural Sciences – Biology Department (Zagreb)	MZOS 0122016	Molecular organisation and function of the photosynthetic apparatus during spruce *Picea abies* (L.) Karst. needles development	Hrvoje Lepeduš	Vera Cesar, Nikola Ljubešić	2003
Faculty of Natural Sciences – Chemistry Department (Zagreb)	MZOS 073-0731674-1673	Lipid peroxidation and antioxidative response during chloroplast biogenesis	Vlatka Gaća	Hrvoje Lepeduš	2006
Faculty of Food Technology and Biotechnology (Zagreb)	MZOS 098-0982913-2838; MZOS 058-0582261-2246	Role of protein TLP40 and TROL in the regulatory mechanisms of oxygenic photosynthesis	Ana Tomašić Paić	Hrvoje Fulgosi, Jasna Franekić	2010
Faculty of Natural Sciences – Biology Department (Zagreb)	MZOS 098-0982913-2838	The role of the gene product At4g01050 in the regulation of photosynthesis in *Arabidopsis thaliana* (L.) Heynh.	Snježana Jurić	Hrvoje Fulgosi	2010
University Postgraduate Inter-disciplinary Doctoral Study of Molecular biosciences (Osijek)	MZOS 073-0731674-1673; MZOS 073-1781844-1930	Sour cherry (*Prunus cerasus* L.) genetic variability and photosynthetic efficiency during drought	Marija Viljevac	Hrvoje Lepeduš Zorica Jurković	2012
Faculty of Natural Sciences (Tuzla, Bosnia and Herzegovina)	MZOS 073-0731674-1673	The regulatory role of temperature in biogenesis of photosystem ii in cotyledon plastids of sunflower (*Helianthus annuus* L.) during transformation ethyoplast to chloroplast	Sanida Osmanović	Hrvoje Lepeduš	2013
University Postgraduate Inter-disciplinary Doctoral Study of Molecular biosciences (Osijek)	MZOS 073-0731674-0841; MZOS 073-0731674-1673	The extreme drought effect on biochemical and physiological features of barley (*Hordeum vulgare* L.) seedlings grown under the low and high light	Jasenka Antunović	Vera Cesar, Hrvoje Lepeduš	2013
University Postgraduate Inter-disciplinary Doctoral Study of Molecular biosciences (Osijek)	MZOS 073-0731674-1673; MZOS 073-0731674-0841	Photosynthetic efficiency and antioxidative response in young and mature leaves of common fig (*Ficus carica* L.) during light stress	Selma Mlinarić	Hrvoje Lepeduš, Vera Cesar	2013

Table 2. Continued.

POSTGRADUATE STUDY or INSTITUTION	PROJECT(S) CODE	DISSERTATION / M. Sc. THESIS TITLE	CANDIDATE	MENTOR(S)	THE YEAR OF DEFENCE
Faculty of Natural Sciences (Tuzla, Bosnia and Herzegovina)	MZOS 073-0731674-0841	Biochemical, physiological and anatomical changes in Norway spruce (*Picea abies* L. Karst.) needles pronounced by urban dust pollution in the area of Tuzla city	Samira Huseinović	Vera Cesar	2013
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences (Osijek)	IP-2014-09-1173	Functional analysis of PEPE domain in TROL protein from *Arabidopsis thaliana* (L.) and its role in the regulation of photosynthesis	Renata Hanzer	Hrvoje Fulgosi	2016
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences (Osijek)	MZOS 073-0731674-1673; MZOS 073-1781844-1930	Adjustment of photochemical mechanisms to environmental stress affected by increased temperature and excess irradiation in apple cultivars	Ines Mihaljević	Hrvoje Lepeduš	2016
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences (Osijek)	IP-2014-09-4359	Plant stress response of Brassicaceae upon increased salinity: the role of auxin and stress hormones	Iva Pavlović	Branka Salopek-Sondi	2017
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences (Osijek)	HRZZ-IP-2013-11-5707	Effects of cadmium on photosynthetic parameters in different maize genotypes	Mario Franić	Domagoj Šimić	2018
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences (Osijek)	MZOS 073-0000000-3538; MZOS 073-0731674-167	Biochemical regulatory mechanisms of photosystem II efficiency in sunflower leaves affected by the environmental stress	Antonela Markulj Kulundžić	Hrvoje Lepeduš Vera Cesar	2019
University Postgraduate Interdisciplinary Doctoral Study of Molecular biosciences (Osijek)	MZOS 073-0731674-0552; MZOS 073-0731674-167	Regulatory mechanisms of photosynthetic efficiency in soybean genotypes during different developmental stages under the influence of drought stress	Ana Josipović	Hrvoje Lepeduš Josip Kovačević	2019

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Part 2

Physiological Aspects of Chlorophyll *a* Fluorescence Application

Chlorophyll *a* Fluorescence in Evaluation of Plant Responses to Environmental Signals

Željka Vidaković-Cifrek and Mirta Tkalec

Ever-changing environmental conditions affect the structure and functionality of membranes, protein complexes, and molecules involved in the photochemical reactions of photosynthesis. Two types of photosystems (PSI and PSII) play important roles in the absorption, transfer, and conversion of light energy. The variable part of chlorophyll fluorescence mainly originates from PSII, thus reflecting the structural and functional properties of PSII and its photochemical efficiency. Because PSII significantly affects the overall dynamics of the electron transport chain, the assessment of its functionality by measuring the kinetics of the chlorophyll *a* fluorescence (ChlF) signal could be considered an indicator of environmental influences on primary photosynthetic reactions (*Strasser et al. 2000, Murchie and Lawson 2013, Barboričová et al. 2022*).

Changes in physiological and biochemical parameters in plants in response to environmental factors have been studied at the Department of Biology, Faculty of Science, University of Zagreb, since 2007, when the FL2LP Chlorophyll Fluorescence Package (Qubit Systems Inc., Canada) was purchased. The first experience with pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method was gained on aquatic plant duckweed (*Lemna minor* L.) exposed to heavy metals, in collaboration with Prof. Hrvoje Lepeduš (*Tkalec et al. 2006*). It has been known for a long time that heavy metals are present in the environment due to natural processes and human activities, so their effects on photosynthetic performance and other physiological and biochemical processes in plants have been studied by many authors on numerous plant species (*Aravind and Prasad 2004, Parmar et al. 2013, Paunov et al. 2018*). Most of these studies focused on the effects of plant exposure to a single heavy metal, while less attention was paid to the simultaneous effects of multiple metals. In our work (*Vidaković-Cifrek et al. 2015*), we investigated the effects of the single metals Zn, Cd, and Cu, as well as the interaction of Cd with Zn or Cu in duckweed plants. The results showed a pronounced inhibitory effect of the addition of Cu alone and of Cu in combination with Cd on maximum quantum yield (F_v/F_m), and the effective quantum yield of PSII photochemistry (Φ_{PSII}), while the treatment with Zn or Cd alone and the combination of Cd with Zn moderately affected the observed parameters. The more pronounced inhibitory effect of treatments with Cu, compared to treatments with Zn, on PSII efficiency is probably due to the ability of Cu to enhance the formation of reactive oxygen species through its redox activity (*Babu et al. 2001*).

In recent years, the presence of metals in the form of nanoparticles, including silver nanoparticles (AgNPs) (*Martínez et al. 2020, Tortella et al. 2020*), has been recognised as a potential global problem, and research on the effects of these particles on plants has been initiated (for a review, see *Tkalec et al. 2019, Biba et al. 2022a*). At the Department of Biology, Faculty of Science, University of Zagreb, a model plant, *Nicotiana tabacum* L., was used to study the mechanism of toxicity of AgNPs in plants. The results showed that the effect of nanoparticles is greater than that of the ionic silver form and that the induction of oxidative stress is the main mechanism of phytotoxicity of AgNPs (*Biba et al. 2022b*). In addition, deterioration of PSII activity was observed, along with reduced pigment content and changes in chloroplast ultrastructure, but the severity of effects differed depending on the type of AgNPs used (*Peharec Štefanić et al. 2021*), as well as plant age (*Biba et al. 2021*). The effects of AgNPs were also studied in *L. minor*, where different physiological and biochemical changes were found, depending on the concentrations used (*Glavaš Ljubimir et al. 2023*). Considering photosynthetic parameters reduced pigment content and decreased F_v/F_m and Φ_{PSII} were observed. Moreover, inhibition of growth, induction of oxidative stress, accumulation of silver, and changes in nutrient content in plant tissue were noticed.

ChlF was also used as one of the methods for investigating whether electric arc furnace slag, generated as a by-product during steel production, could be used as a soil enhancer in agriculture (*Radić et al. 2022*). Together with many other parameters analysed on common bean (*Phaseolus vulgaris* L.), the functionality of PSII estimated by the ChlF method confirmed that electric arc furnace slag applied at low levels had promising potential as a valuable source of nutrients essential to plants.

In addition to evaluating potential environmental pollutants on photosynthetic performance, the influence of other environmental factors was also studied. The effect of different conditions on the growth and photochemical efficiency of PSII was estimated by exposing *L. minor* to different light sources and sucrose supply (*Vidaković-Cifrek et al. 2013*). Among the groups of plants exposed to two types of light sources, plants illuminated with light whose spectral distribution more closely matched the action spectrum of photosynthesis showed better photosynthetic efficiency. The adaptation of duckweed to different light conditions was further studied using the saturation pulse method as well as ChlF transient induction and the JIP test (*Lepeduš et al. 2020*). In plants exposed to low light conditions, overall photosynthetic performance decreased, which was greatly reflected in biomass production. The decreased total chlorophyll and carotenoid content in plants exposed to high light indicated a long-term acclimation response to the increased irradiance. In addition, plants exposed to high light showed a better ability to use absorbed light in photosynthesis, accompanied by a photoprotective adjustment of a certain number of PSII reaction centres.

Besides being used in studying the effects of abiotic stress on the photosynthetic process in various C3 plants, the analysis of ChlF was employed in studying differences in photosynthesis between C3 and C4 plant species under stress conditions (*Guidi et al. 2019* and references therein), and in CAM plants under changing environmental conditions (*Ceusters et al. 2019* and references therein). F_v/F_m was the first and most commonly used indicator of plant photosynthetic performance in these studies, although other parameters such as coefficients of photochemical and non-photochemical quenching (qP and qN), Φ_{PSII}, and many others have also been used to gain more insight into the photoprotection mechanism of PSII (*Guidi et al. 2019*). More recently, the analysis of the fast ChlF induction was applied to investigate metabolic plasticity of CAM, and the performance index (PI_{ABS}), which quantifies the overall functionality of electron flow through PSII, was found to be a sensitive parameter to detect stress in CAM plants (*Ceusters et al. 2019*). At the Department of Biology, Faculty of Science, University of Zagreb, as part of extensive research on the biochemical properties of the cactus *Mammillaria gracilis*, ChlF parameters were measured using the saturation pulse method to determine the influence of *in vitro* culture on the functionality of the photosynthetic apparatus in this CAM plant (*Balen et al. 2012*). Interestingly, all tissues grown *in vitro* exhibited the typical CAM physiology, although photosynthesis was downregulated as a result of sugar supplementation. The most prominent changes found in hyperhydric and tumour tissues were associated with the loss of characteristic tissue organization pattern.

ChlF, together with other photosynthetic parameters, has also been widely used to understand the specific physiology of carnivorous plants. Carnivory involves a complex interplay between nutrient uptake from prey on the one hand, and photosynthetic efficiency, as well as growth rate and reproductive success on the other (*Pavlovič and Saganová 2015*, *Adamec and Pavlovič 2018*). At the Department of Biology, Faculty of Science, University of Zagreb, we investigated photochemical efficiency in *Sarracenia*, a genus of carnivorous plants with leaves modified into pitchers (*Tušek et al. 2016*). The lowest values of F_v/F_m and

F_v/F_0 parameters, as well as chlorophyll content, were found in the red-coloured operculum and the upper part of the pitcher, with numerous nectar-secreting glands reflecting their role in attracting and capturing prey, while the highest photochemical efficiency and chlorophyll content were recorded in the green-coloured wing, a photosynthetically active part of the leaf. We also studied the mechanism of photosynthetic plasticity in *Drosera rotundifolia* plants, which usually grow in sun-exposed peat bogs but can also adjust to a shadier environment. Sun-acclimated plants were characterised by a higher F_v/F_0 ratio and a higher carotenoid/chlorophyll (Car/Chl) ratio, while plants acclimated to low-light conditions had higher chlorophyll content, but surprisingly there was no difference in the photochemical efficiency of PSII (*Tkalec et al. 2015*). Sudden exposure of plants acclimated to low-light to solar radiation resulted in an initial inhibition of PSII activity, which was restored after a 7-day exposure. This correlated with decreased chlorophyll content and increased Car/Chl ratio, revealing that sundews can successfully acclimate to both low- and high-light intensities by changing photosynthetic pigment content and composition.

In addition to its application in higher plants, ChlF has been widely used as a non-destructive method for measuring photosynthetic activity as an indicator of the health status of lichens, a symbiotic association between fungi and certain groups of cyanobacteria or unicellular green algae. Determination of the F_v/F_m parameter is a relatively rapid method for analysing large numbers of specimens, and because this parameter is not temperature sensitive, it has been suggested as an ideal tool for monitoring lichen vitality in both the laboratory and the field (*Jensen 2002*). In lichen photobionts, F_v/F_m ranges from 0.63 to 0.76, which is lower than in higher plants, where it is around 0.83 (*Jensen 2002*). Measurement of F_v/F_m has been used to assess the health of lichen in the Himalayas (*Nayaka et al. 2009*) and impacts of metal pollution (*Maslać et al. 2016* and references therein), for biomonitoring in air pollution studies (*Jensen 2002* and references therein), as well as to investigate the susceptibility of lichens to photoinhibition under stress (*Gasulla et al. 2012*). In our laboratory, we investigated the effects of short-term Cd-exposure on the photosynthetic performance of three widespread epiphytic lichen species: *Parmelia sulcata*, *Flavoparmelia caperata*, and *Evernia prunastri,* to assess their sensitivity to metal pollution. The values of F_v/F_m were between 0.625 and 0.56, while treatment with cadmium caused a decline, but only after 8 days (*Maslać et al. 2016*).

Due to climate change, there is considerable interest in the effects of heat and water deficit on plants (*Hasanuzzaman et al. 2013, Kreslavski et al. 2023*). Plant growth and productivity are highly dependent on photosynthesis, and photosynthetic efficiency is considered in many studies of the negative effects of these environmental factors (*Sharma et al. 2020, Barboričová et al. 2022*). In our ongoing study, measurement of ChlF using the fluorometer PAR-FluorPen FP 100-MAX-LM (Photon Systems Instruments, Brno, Czech Republic) is used to estimate the effect of moderately increased temperature, salinity, and osmotic stress on *Arabidopsis thaliana* seedlings. In addition to the wild type, a line with modified expression of BPM proteins involved in the regulation of the ubiquitin-proteasome pathway of protein degradation (*Morimoto et al. 2017, Škiljaica et al. 2022, Vitko et al. 2022*) is also included. The ChlF induction kinetics followed by a JIP test showed that wild type seedlings and the seedlings overexpressing the *BPM1* gene exhibited a decrease in PI_{ABS} and F_v/F_m compared with nonstressed seedlings, regardless of the stress conditions applied. In seedlings exposed to stress conditions, especially heat stress, a decrease in the number of active reaction centres of the photosystem II (RC/CS_0) was observed, and the previously mentioned decrease in PI_{ABS} could be the result of a lower amount of RC/CS_0. The JIP test showed no difference between the wild type seedlings and the line overexpressing the *BPM1* gene in terms of response to the stress conditions studied (*Vitko*, unpublished results).

In addition to the model plant *A. thaliana*, the effects of elevated temperature and drought stress were studied in the agronomical important plant kale *Brassica oleracea* var. *acephala* (*Bauer et al. 2022*). A wide range of biochemical changes, as well as ChlF induction kinetics, were measured to evaluate the tolerance mechanisms of 33 local kale accessions to single stress factors (drought or heat), as well as to the simultaneous application of these two stress factors. When comparing the obtained results, the PI_{ABS} parameter proved to be more sensitive to the applied abiotic stress factors than F_v/F_m. An interesting observation was the response of plants to a combination of stress factors (high temperature and drought stress), which caused less disturbance to photosynthetic performance than drought stress alone and did not result in a significant decrease in PI_{ABS} or F_v/F_m parameters.

Besides the environmental factors mentioned so far, there are a variety of mechanical stimuli that plants perceive and respond to. Examples include strong wind, frost, hail, flooding, soil movement, and trampling by animals. Like other environmental factors, mechanical stimuli affect various biological processes at the tissue and cellular levels, including photosynthesis (*Chehab et al. 2009, Li and Gong 2011, Monshausen and Haswell 2013, Gardiner et al. 2016, Reis et al. 2018, Zhdanov et al. 2022*). In our laboratory, we successfully applied an external, precisely quantified, moderate mechanical force to *A. thaliana* leaf blades and monitored selected physiological and biochemical changes. Our results indicate that plants perceive a moderate force, but that it does not induce pronounced changes in metabolism or photosynthetic performance. Considering the results of the saturation pulse method, the activity of PSII was slightly affected – there was no significant difference in F_v/F_m value between the control and stimulated leaves, while Φ_{PSII} was decreased in the stimulated leaves 20 hours after treatment (*Šutevski et al. 2023*).

Changes in environmental conditions are not always detrimental and stressful to plants, sometimes they can have only a mild effect. The high sensitivity of chlorophyll fluorescence methods makes them suitable for detecting the response of plants to environmental signals in a short time. In comparison, some other indicators of environmental changes, such as growth rate and plant pigment composition, can only be detected after a longer period of time. Another advantage of fluorescence measurement techniques is their applicability under *in vivo* conditions, allowing the same plant material to be used in other analyses, such as quantifying compounds that are indicators of the effect of environmental conditions or repeating measurements over a longer period of time.

In addition to scientific research, the evaluation of photosynthetic efficiency by ChlF measurement is a part of the teaching activities at the Department of Biology, Faculty of Science, University of Zagreb. Since 2010, chlorophyll fluorescence as a phenomenon and the application of the saturation pulse method in plant research has been demonstrated in the elective course "Structure and Function of Photosynthetic Membranes" at the graduate study program of Molecular Biology. Prof. Hrvoje Lepeduš (J. J. Strossmayer University of Osijek), who brought his own equipment (PAM, Walz, Germany), usually cooperated in the implementation of this part of the course. Today, the title of the course is "Regulatory Mechanisms of Photosynthesis", but the chlorophyll fluorescence – saturation pulse method and chlorophyll *a* transient induction followed by JIP test are still an important part of the course.

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Changes of Chlorophyll *a* Fluorescence Parameters Influenced by Light and Temperature Stress

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Introduction

Plants are exposed to various stressors that act together in their natural environment, and intense irradiations combined with increased temperatures are the most frequently experienced stresses under field conditions. When changes in environmental conditions exceed plant capacity for acclimation, photoinhibition occurs (*Nishiyama et al. 2011*). As a result, plants display decreased quantum yield of photosystem II (PSII) and disturbed photochemistry (*Takahashi and Murata 2008, Tyystjarvi 2008*). The chlorophyll *a* fluorescence (ChlF) has been extensively used as a non-invasive, very sensitive, and fast method for the estimation of the photosynthetic performance that can provide a reliable source of information on plant conditions (*Begović et al. 2016, Goltsev et al. 2016, Mlinarić et al. 2017, Kalaji et al. 2018, Swoczyna et al. 2019, Begović et al. 2020, Mlinarić et al. 2021a, Mlinarić et al. 2021b*). However, the accuracy and reliability of such measurements can be influenced by various environmental factors, including light and temperature. To broaden the understanding of the underlying mechanisms involved, various parameters derived from the JIP test can be used to describe the influence of light and temperature on photosynthetic performance. It can give us information about the tolerance to light and temperature stress, as well as on the utilization of light in photosynthetic apparatus (*Müller et al. 2001, Strasser et al. 2004*). To reveal the dynamics of photosynthetic incidence, various parameters can be used. The most common parameters and most sensitive ones are the performance index on absorption basis (PI_{ABS}), and total performance index (PI_{total}). They include the most important functional steps of primary photochemistry and, consequently, the vitaliy of photosynthetic units (*Strasser et al. 2004, Yusuf et al. 2010, Krüger et al. 2014*). Nevertheless, numerous other parameters could be used to analyze the functionality of PSII and the limitations of electron transport at different levels of the electron transport chain.

Light

Light is the primary energy source for photosynthesis. The intensity, quality, and duration of light – all affect the rate of photosynthesis and its efficiency. In the natural environment, plants are exposed to fluctuations in the quantity and quality of the incident light. They can adjust the physiological and biochemical processes to sudden changes in light conditions. Moreover, plants have evolved to capture light energy efficiently, and their photosynthetic apparatus is optimized to make the most of the available light. In conditions when light is in excess, part of the absorbed energy cannot be efficiently used for photosynthesis, and it is dissipated as heat or as fluorescence (*Müller et al. 2001, Nishiyama et al. 2011*).

High light intensity is a common environmental stress factor that can negatively impact the photosynthetic performance of plants. Light intensity refers to the amount of light energy that falls on a unit area of a plant's surface. Processes like photoinhibition, photooxidation, or photoinactivation usually occur in high light intensity conditions which declines photosynthetic capacity (*Janeeshma et al. 2022*). At the high intensity of light, the key photosynthetic protein, D1, positioned in the reaction center (RC) of PSII, gets phosphorylated, and its turnover is the most important feature of recovery after photoinhibition. In high light conditions, D1 turnover is usually inhibited or decreased and mostly accompanied by damage to the oxygen-evolving complex (OEC) (*Murata et al. 2007, Cheng et al. 2016, Gupta 2020*). To deal with excess light conditions, plants develop various adaptations to protect themselves from photoinhibition (*Kalaji et al. 2012*).

Due to nonfunctional PSII in such conditions, photosynthetic efficiency reduces while most of the absorbed light energy is dissipated non-photochemically as heat. Usually, an increase in non-photochemical quenching, NPQ (*Murata et al. 2012, Hazrati et al. 2016)*, or dissipation of excess energy per active RC (DI_0/RC) (*Bayat et al. 2018, Ceusters et al. 2019*) were shown to be an efficient mechanism to decrease excitation pressure on PSII and reduce the damage to photosynthetic apparatus. As a result, overall photosynthetic performance (PI_{ABS} and PI_{total}) decreases (*Kalaji et al. 2012, Janeeshma et al. 2022)*. Numerous publications refer to the negative influence of high light exposure on PI_{ABS}, such as on two barley cultivars with differing susceptibility to stress *(Kalaji et al. 2018*), rose plants grown at different light spectra (*Bayat et al. 2018*), or on young and mature fig leaves during the day course (*Mlinarić et al. 2016, Mlinarić et al. 2017*). Except for the performance index (PI_{ABS}) short-term high light intensity was shown to decrease structure-function index (SFI_{ABS}), the area above the fluorescence curve describing the size of the reduced pool of plastoquinone (Area), as well as maximum quantum yield (ϕP_0) and electron transport quantum yield (ψE_0) in three rice cultivars, which makes them reliable parameters for distinguishing tolerant and sensitive rice cultivars (*Faseela and Puthur 2017*). Decreased Area was also reported as the parameter that could help in the identification of early light stress in barley (*Kalaji et al. 2012*).

It was reported that parameters F_0 and F_m, denoted as minimal and maximum fluorescence intensity, could also be reliable parameters to detect light stress. Increased F_0 is the result of disconnected light-harvesting complex (LHC) antennae from PSII (*Mathur et al. 2018*), while the decreased F_m reflects the damage of the PSII donor side, especially OEC (*Faseela and Puthur 2017*), both of which lead to decreased electron transport rate. A decline in electron transport is usually the result of degraded and/or damaged thylakoid membranes (*Mlinarić et al. 2016, Janeeshma et al. 2022*), whose intactness is necessary for efficient electron transport activity. Likewise, energy fluxes per active RC, electron transport (ET_0/RC), trapping (TR_0/RC), absorption (ABS/RC), dissipation (DI_0/RC), and PSI reduction (RE_0/RC) can be used to quantify the efficiency of energy transfer and electron transport in the photosynthetic apparatus. High light intensity usually increases ABS/RC due to a decrease in antenna size, which consequently reduces the amount of trapped energy by RCs (TR_0/RC), resulting in reduced quinone A (Q_A). Reduced Q_A^- unable to oxidize cannot be efficiently used in the photochemical reaction and electron transport (decreased ET_0/RC) but dissipates as heat (increased DI_0/RC) (*Mlinarić et al. 2017, Bayat et al. 2018, Kalaji et al. 2018, Janeeshma et al. 2022*).

The shape of OJIP transients can also give us information on the status of the plant. It was shown to be a good indicator of the pool size of the electron carriers in the electron transport chain. Therefore, when plants are exposed to various stressful conditions, the shape of the OJIP curve can change, and the intensity of specific steps changes (*Strasser et al. 2010, Kalaji et al. 2018*). A recent study in rose plants grown at different light spectra showed a decrease in fluorescence intensity at all steps of OJIP transient after exposure to high light intensity (*Bayat et al. 2018*). Depending on the tolerance of a specific genotype, prolonged exposure to high light intensity usually induces changes to OJIP steps intensity. *Kalaji et al. (2018)* showed that 7-day exposure decreased the intensity of all steps in the OJIP curve in two barley landraces.

Low light intensity usually induces different adaptations in plants than in high light conditions. While at high light conditions, plants have to protect themselves from photoinhibition, at low light, they have to adjust their functioning to use the available light efficiently for optimal photosynthesis. Usually, thylakoid, photosystem, pigment, and/or protein adjustments at the structural level occur (*Lichtenthaler et al. 2007, Kouřil et al. 2013, Zivcak et al. 2014*). An increased leaf area and chlorophyll content are also common features of low light grown plants that enable them to capture more incident light (*Zhang et al. 2016*). Also, low light induces alterations in the photosynthetic apparatus resulting in the limitation of electron transport due to the lower amount of electron carriers and lower connectivity of PSII units in shaded leaves (*Desotgiu et al. 2012, Zivcak et al. 2014*).

Plants growing at constant low light usually exhibit less efficient photosynthetic reactions compared to plants grown at fluctuating light in natural conditions, even though their maximum quantum yield (F_v/F_m) was over 0.8 (*Janeeshma et al. 2022*). It was recently suggested that such results were possible due to acclimatization to suboptimal light conditions (*Torres et al. 2021*). However, it was also suggested that C3, C4, and CAM types of plants would exhibit differential acclimatization mechanisms to such responses. Furthermore, low photosynthetic efficiency, namely F_v/F_m, is more pronounced in younger leaves than in mature ones, and that decrease in F_v/F_m was regulated by the efficiency of the Calvin cycle (*Zhang et al. 2016)*. Moreover, it was shown that maintaining continuously low F_v/F_m at low light makes plants more sensitive to low light stress.

Recent investigation on tomato plants exposed to low light for seven days revealed lower PI_{ABS}, quantum yield of absorbed photons for electron transport (ϕE_0), and quantum yield of reduction of final electron acceptors of PSI per photon absorb (ϕR_0), while ABS/RC, TR_0/RC, and DI_0/RC, as well as maximum yield of primary photochemistry (ϕP_0), increased (*Lu et al. 2019*). Our recent investigation on invasive species of Japanese knotweed (*Reynoutria japonica* Houtt.) grown at continuous low light showed less efficient photosynthetic reactions compared to plants grown at fluctuating light. Also, low light grown plants revealed lower grouping and connectivity (L band, lower overall grouping probability for the use of the absorbed energy in photochemical reactions (P_{2G})) between the PSII units, while OEC (K band, lower maximum primary yield of the PSII photochemistry (F_v/F_0)) was shown to be less functional than in plants at fluctuating light. Furthermore, low light grown knotweeds revealed higher ABS/RC, TR_0/RC, and DI_0/RC, while ET_0/RC and RE_0/RC were lower, suggesting less efficient electron transport due to a lower plastoquinone pool. Moreover, measurements and analysis of modulated 820 nm reflection transients revealed slower oxidation at PSI side in low light grown plants. Such results suggested that plants at fluctuating light generated the cyclic electron flow around PSI as an efficient adaptive mechanism for minimizing the photooxidative damage by regulating the distribution of excitation energy between PSII and PSI (*Mlinarić et al. 2021a*).

The quality and duration of exposure to light also influence photosynthesis. Different wavelengths of light have different energies, and plants have specialized pigments to capture different wavelengths of light. Chlorophyll, the primary pigment involved in photosynthesis, absorbs light in the blue and red parts of the spectrum most efficiently. Other pigments, such as carotenoids and phycobilins, capture light in other parts of the spectrum (*Serrano-Bueno et al. 2017, Roeber et al. 2022*). The prolonged exposure to light affects key components in photosynthetic processes, namely influences the excitation of PSI and PSII (*Islam et al. 2021*). Photosynthetic capacity is increased by longer photoperiods which in turn induces growth (*Gendron et al. 2021*). However, numerous negative and adverse effects can also be induced in conditions of continuous light or prolonged photoperiods, such as chloroplasts with smaller grana stacks and increased chlorophyll content (*Sysoeva et al. 2010, Roeber et al. 2022*). The most common result of prolonged exposure to continuous light is disturbed and/or damaged OEC mirrored in the positive K band (*Kumar et al. 2020, Šrajer Gajdošik et al. 2022)*. However, in such conditions plants developed an efficient strategy to protect themselves using alternative donors (*Kumar et al. 2020*). In addition, higher dissipation in the form of heat was also reported as an effective protection mechanism from photoinactivation (*Lee et al. 2005, Kalaji et al. 2018, Šrajer Gajdošik et al. 2022*).

The investigation of different photoperiods (16/8, 20/4, and 24/0) on two *Cannabis* cultivars grown at white and purple light showed diverse photosynthetic reactions. The most beneficial condition was the 16/8 photoperiod, regardless of the light type, since it brought the most efficient physiological response and inducing. However, different efficient adaptation strategies were employed based on the type of light and the duration of the photoperiod. White light at both photoperiods caused higher dissipation (DI_0/RC) of excess light, causing reduced pressure on PSI. Purple light in 20/4 grown *Cannabis* induced efficient dissipation of excess energy and formation of cyclic electron transport around PSI, suggesting an efficient repair system, while 24/0 grown plants maintained functional electron transport between two photosystems (ET_0/RC, δR_0), suggesting a positive effect on the photosynthetic reactions (*Šrajer Gajdošik et al. 2022*).

Temperature

During evolution, plants have developed long and short-term adaptations and adjustments of various structural features and molecular mechanisms to protect the photosynthetic apparatus from damage caused by high and low temperature stress, one of them being the acclimation of PSII. One of the most detrimental stresses of the environment is the high temperature. Predictions show rising in the global air temperature by 0.2 °C per decade, leading to 1.8–4.0 °C higher temperatures by 2100 *(Sharkey and Schrader 2006, IPCC 2007*). As plants are sessile organisms, their processes of growth and development are significantly affected by high temperatures (*Lobell and Field 2007*). Among all plant cell processes, photosynthesis is considered the most sensitive to high temperatures (*Crafts-Brandner and Salvucci 2002, Yang et al. 2006*), with particular emphasis on photosynthetic apparatus whose inhibition involves PSII. There are two main aspects of PSII electron transport inhibition by high-temperature stress, one is the dislocation of PSII light-harvesting complexes from the thylakoid membrane, and the other one is the electron dynamics of PSII integrity (*Mathur et al. 2014*). Furthermore, in different species, PSII sensitivity to stress differs and depends on the organization and acclimation level of PSII (*Janka et al. 2013*). On the other hand, plants exposed to increased temperature display inhibition of OEC and reaction centers of PSII. The OEC is the most temperature-sensitive component of photosynthetic apparatus, and even a slightly elevated temperature causes its deactivation (*Allakhverdiev et al. 2008*). Moderate heat stress was shown to inhibit the repair of damaged PSII, which accelerates photoinhibition (*Takahashi and Murata 2008*). Also, increased temperature impairs the biosynthesis of total chlorophylls and accelerates their degradation (*Ashraf and Harris 2013*).

Low temperature stress implies chilling stress with temperatures 0 °C < 15 °C or freezing with temperatures below 0 °C. Low temperatures are also one of the main factors that limit growth and development, as well as the geographical distribution of plant species (*Allen and Ort 2001, Hasdai et al. 2006*). It was shown that exposure to low temperatures impacts the fluidity of membranes which inhibits electron transport, hence protein mobilization, D1 protein turnover, as well as redox homeostasis (*Aro et al. 1990, Allen and Ort 2001*), and Calvin-Benson cycle (*Ensminger et al. 2006, Horton 2012, Khanal et al. 2017*). This has several consequences, in other words, the induction of stress response by increasing the cyclic electron transport around photosystem I (PSI) (*Asada 1999, Endo et al. 2005*) and the generation of reactive oxygen species (ROS) (*Triantaphylidès and Havaux 2009*) results in photoinhibition (*Tyystjärvi 2013*) of both PSs.

Measurements of ChlF can provide information on processes in PSII that are associated with light energy conversion to a stable chemical form (*Jee 2005, Stirbet 2011*). Different fluorescence parameters have been demonstrated to be good stress indicators for both low and high temperatures. The effects can be detected by observing fluorescence transients (*Roháček et al. 2008*). Induction of OJIP transients can be deciphered through JIP test into several phenomenological and biophysical parameters (*Strasser and Strasser 1995, Strasser et al. 2000, Strasser et al. 2004*) by reflecting the activity of the photosynthetic apparatus and hence quantifying the functioning of PSII (*Strasser et al. 2004*). ChlF parameter F_v/F_m, representing the maximum quantum yield of PSII photochemistry, is one of the most used parameters when evaluating the effect of abiotic stresses. It was shown that high (*Lu and Zhang 2000*) and low (*Mishra et al. 2015*) temperature stress leads to a decrease in F_v/F_m. By observing the OJIP curve, an additional K step could be seen in response to high-temperature stress (45 °C) (*Mathur et al. 2011*), which is associated with inhibition of OEC *(Guisse et al. 1995, Srivastava et al. 1997*), inhibition of electron transport and changes in the structure of the LHC of PSII (*Guisse et al. 1995)*. For evaluation of the impact of low and high-temperature stress on PSII, the performance index (PI_{ABS}) has shown sensitivity in the assessment of the plant response *(Strauss et al. 2006, Kalaji et al. 2011)*. The PI_{ABS} is a multi-parametric expression that involves three steps: light energy absorption, trapping of excitation energy, and conversion of excitation energy to electron transport (*Strasser et al. 2000*).

In their research, *Galic et al. (2019)* studied the response of maize recombinant inbred lines (IRILs) to different heat scenarios, mild and moderate, in two different locations, Croatia and Turkey, respectively. In the mild heat scenario, the temperature exceeded 33 °C during the day and was below 20 °C at night. The moderate heat scenario was characterized by temperatures exceeding 33 °C during the day and higher than 20 °C at night. The authors observed that the efficiency of electron transport beyond quinone A (Q_A) [$ET_0/(TR_0$-$ET_0)$] and performance index (PI_{ABS}) significantly differed between the two heat scenarios. Under the moderate heat scenario, there were close positive genetic correlations between two parameters ($ET_0/(TR_0$-$ET_0)$ and PI_{ABS}) and grain yield. Furthermore, the two locations also showed differences, for the same parameters, with Croatia having higher values than Turkey. The authors concluded that ChlF parameters $ET_0/(TR_0$-$ET_0)$ and PI_{ABS} could possibly contribute to the breeding and selection of maize traits under moderate heat stress since these parameters showed a genetic correlation with grain yield in maize.

Mlinarić et al. (2021c), in their work on common fig (*Ficus carica* L.), explored short-term chilling stress in developing leaves of two common fig varieties. Detached leaves were exposed to low temperature (10 °C) and low irradiation of 50 μmol m^{-2} s^{-1}during 4 h. Both varieties exhibited effective antioxidative activity. L and K bands, describing functional antennae and good connectivity to reaction centers, respectively, showed negative values along with stable D1 protein accumulation. The authors suggested that this indicated functional electron transport of PSII and efficient primary photochemistry. On the other hand, electron flow further than Q_A was blocked as a consequence of limited PSI functionality in both varieties. Variety Zamorčica had a higher decrease in ChlF parameters PI_{total}, $PI_{ABS,}$ and ϕP_0 compared to variety Green matalon, indicating that the Zamorčica variety was more susceptible to photoinhibition to chilling stress than variety Green matalon. Results of the study contributed to the understanding of genotype-dependent response to chilling stress, which is common in spring and can cause solemn injury to thermophilic plant species usually grown and adapted to warmer environments.

Light and temperature

As reviewed, light and temperature are two of the most important factors that influence photosynthesis and thus affect plant growth and productivity. Environmental factors usually act in combination, so high light stress combined with extreme temperature induces photo-oxidation of chlorophyll and down-regulation of photosynthetic efficiency *(Guo et al. 2006*). Increased light intensity and elevated temperature in combination revealed differential damage to photosynthetic pigments, proteins, and thylakoid membranes, depending on the exposure time. Also, extreme conditions usually cause photooxidation of chlorophyll and negatively impact the rate of transpiration and stomatal conductance (*Guo et al. 2006, Janeeshma et al. 2022*). While short-term exposure caused slower and reversible damage occurrence, long-term exposure induced irreversible damage. Damage repair after longer exposure was inhibited due to the formation of ROS *(Murata et al. 2007, Hewezi et al. 2008*).

The combination of high light intensity and increased temperature stress in common fig (*Ficus carica* L.) decreased photosynthetic efficiency and increased the production of reactive oxygen species, which can damage the photosynthetic machinery (*Mlinarić et al. 2016*). Moreover, we discovered that there was a different response between young and mature common fig leaves. While in young leaves, such temperature conditions induced photoprotective strategies characterized by a competent antioxidative system and downregulated photosynthetic activity, mature leaves decreased total chlorophyll content, which maintained a stable Rubisco LSU (Rubisco large subunit) level and a less effective antioxidative system. Our further investigation of combined high light intensity and increased temperature stress at midday (*Mlinarić et al. 2017*) showed that the major difference between young and mature common fig leaves was the differential diurnal accumulation of the main photosynthetic proteins, D1, LHCII, and Rubisco LSU, which was associated with the regulation of photosynthetic activity. Mature leaves showed good connectivity to PSII reaction centers (RCs), shown as negative L band, which enabled more efficient utilization of excitation energy compared to young leaves. Reduced connectivity of LHCII to its RCs (positive L band) in young leaves increased dissipation of excess light and electron transport due to reduced transfer beyond primary acceptor Q_A^- which consequently led to a stronger decrease in overall photosynthetic efficiency at noon. However, efficient recovery after photoinhibition in both leaf types was accomplished, suggesting that mechanisms for acclimation of PSII to high light were present in both leaf types, and those mechanisms were regarded as sufficiently effective in diurnal PSII adjustment.

In a work by *Franić et al.* (*2020*), the authors investigated the effect of different combined types of stress, such as low temperature, heat and severe heat, and low light in maize hybrids. The OJIP curve changed as a response to different treatments, generally at steps J and I. As previously described, the appearance of the K step, as a good indicator of heat stress, was also confirmed in the study. Electron transport and dissipation showed specific reactions to heat and severe heat treatments. Low temperature and low light stress caused specific changes in relative variable fluorescence at J step (V_J) and relative variable fluorescence at I step (V_I). The authors concluded that lower efficiencies of energy conversion were observed as a decrease in cold, heat, and severe heat treatments, respectively.

The impact of different temperatures (10, 20, and 30 °C) on chlorophyll biosynthesis and the maximum quantum yield of PSII (F_v/F_m) in etiolated sunflower cotyledons (*Helianthus annuus* L.) during 24 h was investigated by *Lepeduš et al. (2017)*. The authors observed a reduction in chlorophyll accumulation and the arrest of PSII assembly at lower temperatures (10 °C) compared to 20 and 30 °C. Exposure to short-term increased irradiation of 800 μmol m^{-2} s^{-1} at 20 and 30 °C induced a decrease in effective quantum yield of PSII ($\Delta F/F'_m$) and higher NPQ at 30 °C.

In conclusion, high and low light intensity, increased or low temperature and chilling, as well as their combination, can have a negative impact on the photosynthetic performance of plants, as indicated by changes in JIP test parameters. The JIP test is a useful tool for assessing the fast chlorophyll *a* fluorescence kinetics of PSII and can provide insight into the underlying mechanisms involved in photosynthetic responses to environmental factors. Further research in this area may help to improve our understanding of plant responses to environmental stress factors and facilitate the development of strategies to enhance plant productivity under challenging conditions.

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Exploring the Relationship between Chlorophyll Fluorescence and Drought/Salinity Stress

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In recent decades, drought and salinity have negatively affected plant growth and productivity on a large scale. With climate change, there is an anticipated global increase in the area affected by water scarcity and elevated salinity, intensifying the impact of these environmental stresses on plants (*FAO 2021*). Given the importance of photosynthesis for the sustenance of life, scientists aspire to investigate and improve the structural and functional properties of the photosynthetic apparatus to aid in climate adaptation. Gaining insight into how plants sustain optimal levels of photosynthetic efficiency despite drought and salt stresses is crucial for preserving biodiversity and maintaining plant productivity.

Drought stress

Water deficit conditions can reduce photosynthesis efficiency, which is correlated with reduced water potential and stomatal conductance and decreased relative water content *(Flexas et al. 2016*). The literature on the effect of water deficit on chlorophyll fluorescence parameters is inconsistent, with some studies showing a positive effect, adverse effect, or no effect, depending on factors such as the plant species studied, the type and the duration of stress applied, the soil types, and moisture levels imposed. For example, mild to moderate drought stress can cause stomata closure, leading to a reduction in net photosynthesis to prevent further water loss (*Chaves and Oliveira 2004*). However, this also reduces the supply of CO_2 to Rubisco, promoting its oxygenase function and causing the loss of ATP (*Lawlor and Tezara 2009*). Because of this, plants are unable to use light energy efficiently, causing a breakdown of thylakoid membranes (*Zhu et al. 2021*), alterations in the proportion of photosynthetic pigments (*Li and Kim 2022*), disruptions in the electron transport chain (*Foyer et al. 2012*), elevated generation of reactive oxygen species (*Miller et al. 2010*), all of which are the initial responders to any disruption in plant function (*Stirbet and Govindjee 2011*).

It is widely acknowledged that drought significantly impacts plants' photosynthetic apparatus, as demonstrated by multiple studies (*Goltsev et al. 2012, Jedmowski et al. 2013, Jedmowski et al. 2015, Kalaji et al. 2018, Bashir et al. 2021*). As a result, drought induces various changes, such as altering the redox state of photosystem I (PSI), impairing electron transfer at the acceptor and donor side of photosystem II (PSII), affecting the oxygen-evolving complex (OEC), and reducing energetic connectivity and electron transfer capacity (*Zhou et al. 2019*). Photosystem II is more resistant to drought than PSI, with permanent damage to PSII observed only under extreme drought conditions (*Desotgiu et al. 2012*). Additionally, studies have demonstrated that photosynthesis shows resilience and maintains high stability of the quantum yield of primary photochemistry of PSII when exposed to varying intensities of drought stress (*Oukarroum et al. 2007*, *Oukarroum et al. 2009*, *Qi et al. 2021*). Chlorophyll fluorescence measurements are commonly used to detect the initial non-visible changes in the functioning and structure of the photosynthetic apparatus (*Strasser et al. 2004, Goltsev et al. 2016, Kalaji et al. 2016, Kalaji et al. 2018, Samborska et al. 2019*). Among the obtained parameters for various intensities of drought impact, photosynthetic efficiency indices (PIs) have been proven helpful in screening plants and evaluating the overall effect of stress on photosynthetic performance. *Tsimilli-Michael and Strasser (2013)*, *Zivcak et al. (2014)*, *Kalaji et al. (2017)*, and *Tsimilli-Michael (2020)* argue that the impact on different processes can be inferred from the specificity of the resulting expressions. Moreover, plant drought tolerance can be evaluated using double-normalized differential chlorophyll fluorescence data, namely L (ΔW_{OK}) and K bands (ΔW_{OJ}) (*Oukarroum et al. 2007, Oukarroum et al. 2009, Brestic et al. 2012, Brestic and Zivcak 2013, Guha et al. 2013, Kalaji et al. 2018, Zhou et al. 2019*).

In 2012, Lepeduš and collaborators were the first researchers to explore the relationship between fluorescence and drought in Croatia. Their research was a preliminary inquiry into quantitative trait locus (QTL) analysis of chlorophyll fluorescence parameters to better understand the genetic and physiological basis for drought stress tolerance in maize. Due to genetic and physiological differences, the authors expected that variation in photosynthetic performance under water-limited conditions would develop between flint and dent inbred maize lines. The results demonstrated that, although chlorophyll fluorescence parameters showed similar clustering of inbred lines, differences in photosynthetic efficiency were higher within dent inbred lines (*Lepeduš et al. 2012*). The following article, written by a team at the Agricultural Institute Osijek (Osijek, Croatia), represented by Viljevac et al. (2013) showed that drought is a limiting factor in the production of sour cherries and that two different genotypes of sour cherries have distinct photosynthetic mechanisms for drought tolerance. Their results suggest that the impaired conversion of excitation energy in electron transport caused a decrease in the photosynthetic performance index (PI_{ABS}) and overall photosynthetic efficiency in a drought-sensitive genotype. They also found that electron transport beyond primary plastoquinone acceptor (Q_A^-) expressed per active reaction center (ET_0/RC) was significantly impaired in drought-treated leaves of susceptible genotype due to reduced transport from Q_A to secondary plastoquinone acceptor (Q_B), but this was not the case with the drought tolerant genotype (*Viljevac et al. 2013*). The effect of different soil water content on genotype expression in photosynthetic efficiency was also studied by *Markulj Kulundžić et al. (2016)* but in sunflowers. To assess the drought tolerance of 13 sunflower genotypes, they measured the maximum quantum yield of photosystem II (F_v/F_m), photosynthetic performance index (PI_{ABS}), and leaf temperature. Stress tolerance indices calculated for F_v/F_m and PI_{ABS} confirmed genotype tolerance, and they concluded that these simple indices can assist breeders in selecting sunflower genotypes suited for different farming areas for profitable sunflower production.

Similarly, *Kovačević et al. (2017)* found that specific parameters related to the efficiency of photosynthesis in wheat cultivars can serve as reliable indicators for crucial agronomic traits, especially when measured at an early stage of growth. In their research, ten winter wheat (*Triticum aestivum* L.) cultivars were evaluated for twelve photosynthetic efficiency parameters, as well as water use efficiency and various agronomic traits such as biomass weight, harvest index, yield stability index, and stress tolerance index, to find ways to improve wheat's ability to tolerate drought stress and increase grain yield. During the tillering stage of growth and under drought stress conditions, lower values of absorbed photon flux per excited cross-section of PSII (ABS/CS_0), electron transport flux from Q_A^- to PQ per cross-section of PSII (ET_0/CS_0), and dissipated energy flux per cross-section (DI_0/CS_0), along with higher values of PI_{ABS}, indicated stronger tolerance to drought stress. Conversely, higher values of ABS/CS_0 and ET_0/CS_0 in some cultivars led to increased dissipation (DI_0/CS_0), negatively impacting grain yield, water use efficiency, yield stability, and stress tolerance.

Some aspects of the findings on sour cherries (*Viljevac et al. 2013*) were backed up in new research by *Mihaljević et al. (2021a)*, using progressive drought stress in sweet cherry plants to induce changes in the light phase of photosynthesis. In this research, the authors observed a higher positive L and K bands in the leaves of the modern sweet cherry cultivar, suggesting less connectivity within the PSII units than the autochthonous cultivar and reduced efficiency of OEC due to slower electron flow between OEC and acceptor side of the reaction center. Moreover, the autochthonous cultivar showed an untouched ET_0/RC in drought-stressed leaves, suggesting that this could be the basis of its tolerance and implying that it could protect its photosynthetic apparatus by efficiently utilizing absorbed light energy rather than dissipating it. Therefore, the autochthonous cultivar showed an advantage over the modern one in terms of the efficiency of PSII under progressive drought conditions. *Mihaljević et al. (2021b)* also analyzed the fluorescent transient of OJIP curves in two traditional and one commercial apple cultivar subjected to drought treatment. The chlorophyll fluorescence parameters showed significant differences in leaf photochemistry among the cultivars. For example, the commercial cultivar exhibited higher fluorescence intensity at the J and I steps with the appearance of positive L and K bands compared to traditional cultivars, indicating a more substantial decline and inhibition in electron transport beyond Q_A and PQH_2. Furthermore, the traditional cultivar showed the highest tolerance to drought stress due to a higher amount of active PSII reaction centers and stable absorption flux, whereas the F_v/F_m parameter was not a sensitive indicator of drought stress.

The authors of the following study, *Markulj Kulundžić et al. (2022)*, indicated that drought affected some parameters considerably, such as chlorophyll *b*, the ratio of chlorophylls *a/b*, and the performance index (PI_{ABS}). Moreover, particular chlorophyll fluorescence parameters (relative variable fluorescence at 30 ms (I step) – V_I, the density of reaction centers per excited cross-section – RC/CS_0, average absorbed photon flux per PSII reaction center – ABS/RC, maximum trapped exciton flux per active PSII – TR_0/RC, electron transport flux from Q_A^- to PQ per active PSII – ET_0/RC, and the flux of energy dissipated per active PSII – DI_0/RC) suggested that plants initiated a defense mechanism against water deficiency, meaning that fluorescence measurement could be used for screening large numbers of genotypes in soybean breeding programs to identify superior, adaptable genotypes. However, in elite soybean lines, only chlorophyll fluorescence parameters V_I, TR_0/ABS – maximum quantum yield of primary photochemistry, ABS/RC, RC/CS_0, TR_0/DI_0 - flux ratio trapping per dissipation, and PI_{total}, significantly evaluated variations between soybean genotypes in the photosynthetic apparatus under drought stress, as reported by *Matoša Kočar et al. (2022)*. Further analysis revealed that some parameters could be excluded without losing necessary decision-making information, and they suggested that using TR_0/ABS, ABS/RC, and PI_{total} alone would provide the same amount of data on drought susceptibility as all the other parameters. This would allow the elimination of genotypes with the least efficient photosynthetic apparatus functioning in abiotic stress conditions, boosting breeding efficiency and minimizing costs.

Although a plant's genetics define the molecular, biochemical, physiological, and phenological characteristics that make them susceptible to water deficiency, the severity of these processes is influenced by the plant's water status. *Antunović Dunić et al. (2015)* showed that the cultivation of barley seedlings under high light will induce the adjustment of the photosynthetic apparatus to diminish photo-inhibitory oxidative damages. Photosynthesis was down-regulated, as demonstrated by reduced values of maximum quantum yield of photosystem II (F_v/F_m) and performance index (PI_{ABS}). However, this acclimatization lowered the tolerance of seedlings to the following drought stress, although it was applied under low irradiation. Seedlings showed less efficient and almost dysfunctional photosynthetic apparatus when exposed to extreme drought.

Peršić et al. (2022) conducted a study to distinguish and characterize the reaction of wheat seedlings to simulated physiological drought conditions by identifying the potential photosynthetic processes that explain the diversity among genotypes. The PEG-induced drought mainly caused changes in phenomenological energy fluxes and electron transfer efficiency to final PSI acceptors. Chlorophyll fluorescence parameters, which determine seedlings' response to the imposed drought stress, were grouped into three categories based on the effect size: photochemical parameters related to the donor and acceptor sides of PSII, the thermal phase of the photosynthetic process with electron flow around PSI and the chain of electrons between PSII and PSI, and phenomenological energy fluxes per cross-section. Variable fluorescence parameters at K, L, I steps, and PI_{total} accounted for most of the variations in photosynthetic performance among different wheat genotypes, consistent with previous studies.

Finally, in the following study by *Antunović Dunić et al. (2023)*, the authors described specific reactions and events during the electron flow from PSII to PSI by analyzing prompt chlorophyll fluorescence measurement transients and distinct JIP test parameters to better understand photosynthetic apparatus adaptations to drought, and subsequent recovery by re-watering in selected plant species. The researchers propose that interpreting specific chlorophyll fluorescence parameters could differentiate drought-tolerant and sensitive *Brassica* crops. For example, positive L and K bands, increased PI_{total} and structure-function index (SFI), and positive total driving forces (ΔDF) and drought resistance index (DRI) suggest drought tolerance, while negative L and K bands, lower PI_{total}, SFI, and $RC/CS_{0,}$ indicate lower tolerance or higher sensitivity.

The studies summarized here focus on the relationship between drought stress and chlorophyll fluorescence in various crop species, including maize, sour cherries, sunflowers, wheat, soybeans, and barley. The authors investigated different photosynthetic parameters, such as the maximum quantum yield of photosystem II (F_v/F_m), photosynthetic performance indices (PI_{ABS} and PI_{total}), and electron transport rate (ET_0/RC), to identify genotypes with efficient photosynthetic apparatus, functioning under drought stress conditions. The findings suggest that different crop species exhibit various mechanisms of drought tolerance, and chlorophyll fluorescence parameters can be used to identify genotypes with the efficient functioning of photosynthetic apparatus under drought stress conditions. All studies highlighted the importance of understanding the genetic and physiological basis of drought tolerance in crops to improve crop yield and minimize losses due to water scarcity.

Salinity stress

High salinity induces various salt-specific effects in plants. These effects may include changes in metabolic activity, cell growth, and gene expression at the cellular level, which can have significant implications for plants' survival and adaptation in saline environments. An insight into metabolic activity reveals an initial osmotic adjustment due to water deficiency, but prolonged exposure results in ion toxicity due to excessive uptake of ions such as sodium (Na^+) and chloride (Cl^-). Furthermore, these salt-specific effects can also impact plants' ability to photosynthesize and produce energy. Osmotic stress, for example, will reversibly inactivate photosynthetic electron transport via intracellular space shrinkage, whereas ion toxicity (particularly Na^+) will irreversibly inactivate photosynthetic and respiratory electron transport (*Allakhverdiev et al. 1999, Allakhverdiev et al. 2000, Ashraf and Harris 2013, Isayenkov and Maathuis 2019, Hao et al. 2021*). While some plants can reduce the amount of salt in their cytoplasm, avoiding toxic effects on photosynthesis and other vital metabolic processes, others do not have a mechanism to cope with high salinity levels. These responses are undoubtedly species-specific (*Munns et al. 2006, Munns et al. 2020*).

It is well known that salt stress has a significant impact on the photosynthetic apparatus of various plant species, as shown in a large number of studies (*Chaves et al. 2009, Kalaji et al. 2011, Oukarroum et al. 2015, Khatri and Rathore 2019, Munns et al. 2020, Rastogi et al. 2020, Yang et al. 2020, Shin et al. 2021, Gupta et al. 2022, Maryum et al. 2022, Khalid et al. 2023)*. For instance, research conducted by *Kalaji et al. (2011)* suggests that primary photochemistry may have a significant role in how well barley seedlings respond to salt stress. The authors also demonstrate that the initial salinity effect is closely linked with a restriction of stomatal conductance rather than a reduction of PSII activity. The oxidative stress caused by increased salinity has long been known, and *Oukarroum et al. (2015)* confirmed the correlation between reactive oxygen species (ROS) production and the activities of PSII and PSI. Changes in photosynthetic activity, connectivity of PSII and PSI, and functional antenna size, all of which maintain high photosynthetic performance, have been observed in response to low salinity stress, as reported by *Mehta et al. (2010)* and *Dabrowski et al. (2016)*. In contrast, increased nonphotochemical quenching – NPQ (*Jajoo 2013, Radić et al. 2013*), photoinhibition of PSI and PSII, and the inhibition of the overall electron transport (*Khatri and Rathore 2019, Najar et al. 2019, Zhao et al. 2019*) are typical responses to increased salinity. Moreover, *Mehta et al. (2010)* found that the donor side of PS II was significantly affected, as opposed to the acceptor side.

In a number of studies, the amount of chlorophyll present in a leaf has been measured to understand the effect of salinity on photosynthetic performance (*Radić et al. 2013, Khatri and Rathore 2019, Shin et al. 2021*). Long-term exposure to moderate salinity causes a decrease in the chlorophyll content of pepper (*Zhang et al. 2020*), soybean (*Du et al. 2018*), rice (*Turan and Tripathy 2015*), and lettuce (*Shin et al. 2020*), while exposure to increased salinity causes a reduction in the amount of chlorophyll and carotenoids in cotton genotypes (*Zhang et al. 2014*). An increase in the toxic Na^+ levels was found to be responsible for the observed disorders of chloroplast arrangement in the mesophyll and changes in chloroplast ultrastructure, such as loose granum connections, the enlarged cavity in the thylakoid, and an increase in the number and size of lipid globules (*Barhoumi et al. 2007, Meng et al. 2011, Yang et al. 2011, Shu et al. 2012, Radić et al. 2013*). These changes in chloroplast arrangement and ultrastructure can ultimately lead to a reduction in the efficiency of photosynthesis.

To our knowledge, *Radić et al. (2013)* were the first Croatian authors to investigate plant salt tolerance using chlorophyll fluorescence (ChlF) parameters. The authors tested the effects of salinity (NaCl) and mannitol on the perennial species *Centaurea ragusina* L., which has been recognized as a potential cash crop plant due to a variety of phytochemicals with pharmaceutical or medicinal applications. The treatments were compared to find out if there were any differences in *C. ragusina's* response to ionic (NaCl) and osmotic (mannitol) salinity components. Mannitol-induced osmotic shock resulted in an enhanced downregulation of PSII efficiency more than salinity did, as estimated by decreased values of F_v/F_m, the effective quantum yield of the PSII ($\Delta F/F'_m$), and relative electron transport rate (rel. ETR). Along with PSII downregulation, partial or total inhibition of light-harvesting complexes of PSI and PSII (LHCs) and D1 protein of the reaction center of PSII synthesis has also been reported. The authors also suggested that the downregulation of PSII was caused by a decline in chlorophylls and carotenoid concentration and the presence of modified chloroplasts. Osmotic adjustment, achieved through salt ion uptake and effective antioxidative defense mechanisms, was identified as a dominant strategy for *C. ragusina* tolerance to moderate salinity.

Pavlović et al. (2019) investigated the influence of short-term salinity stress on the photosynthetic performance of three brassicas (Chinese cabbage, white cabbage, and kale). The decrease in total performance index PI_{total}, which describes the overall efficiency of PSI, PSII, and the intersystem electron transport chain, indicated a reduction in the photosynthetic apparatus capacity for efficient energy conversion, particularly in Chinese cabbage, which was the most sensitive of the selected brassicas. Furthermore, the PI_{total} was identified as the most salinity-sensitive parameter and informative stress marker. The same group of authors continued their research by investigating the influence of salicylic and ferulic acid foliar applications on mitigating the adverse effect of short-term salinity on Chinese cabbage (*Linić et al. 2021*). Salicylic and ferulic acids were found to have a protective role in improving photosynthetic performance, as estimated by ChlF parameters such as PI_{ABS}, PI_{total}, ϕP_0 (maximum quantum yield of primary PSII photochemistry), ϕE_0 (quantum yield of electron transport from Q_A^- to PQ), ψE_0 (efficiency with which a PSII trapped electron is transferred from Q_A to Q_B), ϕR_0 (quantum yield of electron transport from Q_A^- to final PSI acceptors), and DI_0/RC (the flux of energy dissipated per active PSII).

Galić et al. (2020) investigated the response of five maize hybrids to salinity stress in the seedling stage by measuring ChlF and fresh and dry mass. The authors incorporated ChlF data into a penalized regression model to predict biomass traits. Their findings described a reasonable proportion of variance in tested phenotypes, demonstrated predictive ability in the independent scenario, and provided physiological context to the obtained predictions. After a comprehensive discussion, they concluded that incorporating chlorophyll fluorescence data into predictive models can improve our understanding of plant responses to environmental stressors and represent a good starting point in crop breeding programs.

The most recent paper in this field of study by *Lazarević et al. (2021)* uses chlorophyll fluorescence and 2D multispectral imaging to quantify changes in the phenotypic traits of basil (*Ocimum basilicum* L.) under moderate (100 mM NaCl) and severe (200 mM NaCl) salinity stress. Under both early and prolonged stress, the anthocyanin index (ARI) and electron transport rate (ETR) were the most influential parameters for differentiating salinity-stressed plants from non-stressed plants.

The studies reviewed here show how functional chlorophyll fluorescence parameters are for understanding plant responses to salinity stress by demonstrating the wide range of applications of chlorophyll fluorescence parameters, from identifying the dominant strategies for salinity tolerance to predicting biomass traits and quantifying phenotypic changes. Thus, further research in this area can help develop more effective methods for crop breeding and management programs in saline environments.

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Effects of Heavy Metals and Metalloids on Photosynthesis

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Introduction

Heavy metals and metalloids (HMs) are characterized by a high density and atomic weight. Although naturally present in rocks, soils, and water, most of them are also released into the environment from various anthropogenic resources such as mining, foundries and smelters, domestic effluents, industry, and agriculture (*Bradl 2005, Okereafor et al. 2020*). Their massive increases in terrestrial and aquatic ecosystems represent a major environmental concern that has consequences for plants, animals, and human health (*Balali-Mood et al. 2021*).

Plants take up HMs primarily via the root system from the soil (*Peralta-Videa et al. 2009*) but also by foliar surfaces through stomata, cuticular cracks, lenticels, ectodesmata, and aqueous pores (*Tangahu et al. 2011, Shahid et al. 2016*). The root uptake is recognized as the main pathway for HMs entry, and it largely depends on the pH value of the soil solution, the organic matter content in the soil, and the concentrations of other ions. The HMs present in the soil bind to the carboxy groups on the root surface, move across the cellular membrane into the root cells, enter into the xylem, and translocate to the aerial parts via symplastic or apoplastic transport (*Seregin and Ivanov 2001, Peer et al. 2005, Pourrut et al. 2011*). Metals are transported from the root as complexes with chelators (organic acids, amino acids, methionine-derivative nicotinamine (NA)), but transport also involves some transport proteins belonging to the families P1B-type ATPases, Multidrug and Toxic compound Extrusion proteins (MATEs), and OligoPeptide Transporter (OPTs) (*DalCorso et al. 2013*).

HMs uptake by foliar pathways varies depending on the physico-chemical characteristics of the metals, as well as on the surface area and texture of the plant leaves, plant habitus (deciduous or evergreen), exposure duration, environmental conditions, and gas exchange (*Beckett et al. 2000a, Beckett et al. 2000b, Souri et al. 2019*). Two foliar pathways of metal uptake have been recognized – diffusion of lipophilic primary elements through the cuticle and of hydrophilic compounds through the aqueous pores of the stomata and via the cuticle (*Larue et al. 2014*). After foliar uptake, metals enter the apoplast, bind to the mesophyll cells, and via the phloem, can be transported towards different plant organs (*Colangelo and Guerinot 2006*). Unlike terrestrial photosynthetic organisms, algae have a large surface area-to-volume ratio, allowing effective contact with the surrounding environment, and may absorb metals firstly by rapid, reversible, and passive adsorption onto the cell surface and then by a slower, irreversible, active process of metal cations transport into the cell cytoplasm across the cell membrane (*Monteiro et al. 2012*). It is enabled by the specific and complex algal cell wall structure containing diverse functional groups (e.g., carboxyl groups, amino groups) that can act as effective binding sites for the HMs (*Spain et al. 2021*).

HMs, including cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), and zinc (Zn), are considered essential elements or micronutrients since they have a crucial role in various metabolic processes at low concentrations (*Peralta-Videa et al. 2009*). However, when the concentrations of non-essential HMs such as cadmium (Cd), lead (Pb), mercury (Hg), chromium (Cr), and metalloid arsenic (As), and selenium (Se) exceed a certain threshold, they produce toxic effects (*Nagajyoti et al. 2010, Ali et al. 2013, Rahman and Singh 2019*). These effects are visible in germination and photosynthetic organism's growth inhibition, physiological disorders, reduction in energy production, the occurrence of oxidative stress, and finally, death (*Prasad and Strzalka 1999, Garg and Singla 2011, Bezini et al. 2019, Cavalletti et al. 2022, Gao et al. 2022, Noor et al. 2022, Nowicka 2022, Pandey et al. 2022*). The accumulation of HMs disrupts chloroplast structure and chlorophyll biosynthesis and negatively affects photosynthesis (*Choudhury and Panda 2004, Pilipović et al. 2019, Ferati et al. 2022*). This process enables photosynthetic organisms to convert the energy of photosynthetic-active radiation into biologically usable energy (*Falkowski and Raven 2007*), and thus, it is directly connected with their growth and viability (**Figure 1**). Most of the light energy absorbed by chlorophyll could be used to drive photosynthesis, but excess energy is usually dissipated as heat or re-emitted as light-chlorophyll fluorescence. These processes occur in competition, so an increase in the efficiency of one will lead to a decrease in the yield of others (*Maxwell and Johnson 2000*). The chlorophyll fluorescence (ChlF) can be determined using the test (JIP) based on the fast rise of *in vivo* ChlF quantum yield after exposure to actinic (i.e. photosynthesis initiating) light (*Strasser et al. 1995, Strasser et al. 2004*). The basic characteristics of these processes are well documented and have been reviewed by different authors (*Stirbet and Govindjee 2012, Schansker et al. 2014*).

ChlF is widely used to evaluate the effect of different kinds of stresses on the photosynthetic apparatus (*Antunović Dunić et al. 2023, Lepeduš et al. 2005, Lepeduš et al. 2009, Mlinarić et al. 2021, Peršić et al. 2022, Šrajer Gajdošik et al. 2022*). Considering the HMs accumulation in the environment, many scientists are focused on the HMs effects on photosynthetic organisms and photosynthetic processes (*Źurek et al. 2014, Štolfa et al. 2015, Begović et al. 2016, Paunov et al. 2018, Žuna Pfeiffer et al. 2018*), and some of the results will be further discussed.

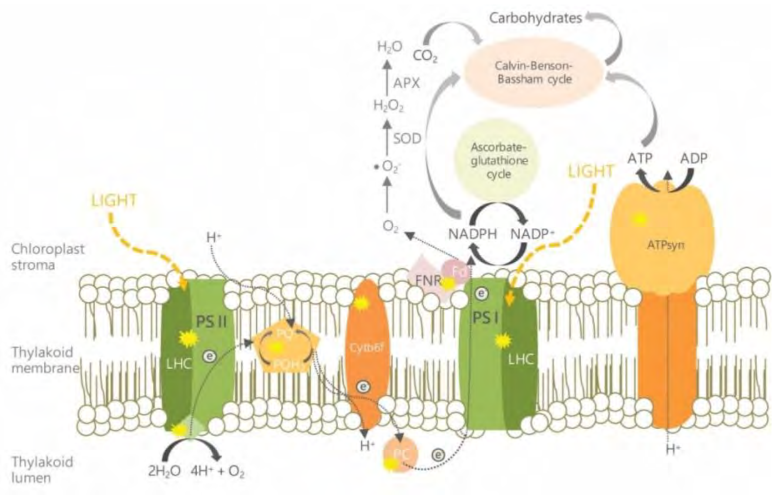

Figure 1. Schematic representation of photosynthesis under HMs stress (source: Špoljarić Maronić D, 2023). *ADP – adenosine diphosphate; ATP – adenosine triphosphate; ATPsyn – ATP synthase; APX – ascorbate peroxidase; Cytb6f – cytochrome b6f complex; e^- – electron; Fd – ferredoxin; FNR – ferredoxin $NADP^+$ reductase; LHC – light-harvesting complexes; NADPH – nicotinamide adenine dinucleotide phosphate; $NADP^+$ – oxidised form of NADPH; PC – plastocyanin; PSI – photosystem I; PSII – photosystem II; PQ – plastoquinone; PQH_2 – dihydroplastoquinone; $\bullet O_2^-$ – superoxide anion; SOD – superoxide dismutase; HMs targets –* ✹

Iron (Fe)

Being an essential and quantitatively important component of electron transfer complexes PSI, PSII, cytochrome b6f complex, ferredoxins, and a cofactor of ascorbate peroxidase (APX) and iron superoxide dismutase (Fe-SOD), Fe is crucial for many cellular processes, including photosynthesis, respiration, and reactive oxygen species (ROS) scavenging (*Raven et al. 1999*). It is also involved in the biogenesis of chlorophylls, hemes, and Fe-S clusters (*Hu et al. 2017*). Photosynthetic cells have evolved several responses to balance Fe supply and demand while minimising the toxicity of excess Fe ions (*Behnke et al. 2023*). Due to the regulation of Fe uptake, high photosynthetic requirement, and limited bioavailability, recent studies focused on the consequences of Fe deficiency on photosynthesis rather than toxicity (*Kroh and Pilon 2020*). Fe deficiency results in a lack of activity in Fe-requiring pathways and influences photosynthetic efficiency by chloroplast degeneration, reduced chlorophyll synthesis, and chlorosis (*Li et al. 2021*), photosynthetic pigment changes (*Ramírez et al. 2013, Tewari et al. 2013, Wang et al. 2023*), altered oxygen-evolving activity and ChlF (*Castell et al. 2022*), and photosynthesis-associated protein regulation (*Lommer et al. 2012, Behnke et al. 2023*). A significant decrease in the total content and activity of PSII was observed under Fe deficit, which can be seen from the decreased effective quantum yield of PSII (Φ_{PSII}) and effective electron transport rate (ETR) (*Tewari et al. 2013*). The maximal quantum yield of PSII (F_v/F_m) decreases in mild and severely chlorotic leaves (*Bertamini et al. 2001*). Fe limitation also promotes changes in the energetics of the recombination reaction between reduced quinone (Q_B) and the S_2/S_3 states of the water oxidation process in PSII (*Castell et al. 2022*). PSI activity is less sensitive to Fe deficiency than PSII. The reduced photosynthetic electron transport in Fe-deficient leaves shows only minor inhibition of PSI activity (*Bertamini et al. 2001*). Fe addition enhances the gas exchange parameters and ChlF. F_v/F_m and Φ_{PSII} increase with the Fe fertiliser foliar treatment, indicating that Fe reduces photoinhibition, promotes photochemical efficiency, and increases non-photochemical quenching (NPQ), coefficient of photochemical quenching (qP), and ETR (*Zhang et al. 2022*). Moreover, Fe treatment improves NPQ levels and photosynthetic electron transport by reducing the excess excitation energy in the reaction center. Plants and algae show changes in photosynthetic activities in response to different Fe valences. Fe^{2+} enhanced photosynthesis parameters, F_v/F_m, and Φ_{PSII}, to a greater extent than Fe^{3+} (*Wang et al. 2023*). The Fe^{2+} has a better-promoting effect on electron transport between the PSII and PSI than Fe^{3+}, with higher ETR_{max} and saturation irradiance (E_k) values, which suggests a higher light intensity tolerance.

Impairment of photosynthetic electron transport in Fe-deprived plants results in electron transfers to O_2, generating excessive levels of $O_2\bullet^-$ and hydrogen peroxide (H_2O_2) accumulation in chloroplasts (*Tewari et al. 2013*). Fe is involved in various ROS metabolic mechanisms, and its concentration is decisive for the amount and activity of ROS. Hence, most of the Fe-regulated ROS metabolism genes, encoding important ROS-scavenging molecules, are up-regulated in the high Fe condition (*Le et al. 2019*).

Excess Fe can increase uptake and toxicity, mainly regulated at the transcriptional level through ferritin gene expression in the chloroplast, one of the most critical mechanisms against oxidative stress (*Kroh and Pilon 2020*). Excessive Fe treatment can increase chlorophyll contents, chloroplast shape, sizes and structure, as well as the number and dimensions of starch granules (*Li et al. 2021*).

Copper (Cu)

Cu is a redox-active HM involved in vital plant functions, most importantly photosynthetic electron transport chain in chloroplasts and the aerobic respiration in mitochondria, and serves as a cofactor of several plant metalloproteins, among which, the most abundant are Cu/Zn-SOD, plantacyanin, and polyphenol oxidase (*Krayem et al. 2021*). Cu deprivation decreases photosynthetic electron transport capacity due to a lack of plastocyanin (*Shahbaz et al. 2015*). Excess Cu is toxic, alters numerous biochemical and physiological processes, and may disturb plant photosynthesis and induce leaf senescence. Cu was found to inhibit photosynthesis due to the reduced photosynthetic pigment content and damage to PSII functions (*Jin et al. 2021*) and chloroplast structure (*Da Costa and Sharma 2016*), altered expression of essential photosynthesis proteins, i.e. PsbD, PsbO, and PsaA (*Wu et al. 2021*) and carbohydrate metabolism (*Zhao et al. 2018*).

Under Cu stress, photosynthesis, gas exchange, and electron transport are inhibited, altering the main ChlF parameters. Excess Cu acts through the inactivation of PSII reaction centers and inhibiting electron transport on the acceptor side (*Xia and Tian 2009*). The decrease in the photosynthetic rate (P_N), transpiration rate (E), and stomatal conductance (g_s), as well as the ChlF kinetics, observed through F_v/F_m and the maximum primary yield (F_v/F_0) of the PSII photochemistry, reflects the photo-inhibitory damage of the PSII reaction centers (*Burzyński and Żurek 2007, Da Costa and Sharma 2016, Shi et al. 2020*). The absorption of fluorescence quanta per PSII reaction center (ABS/RC) increases, while electron transport at time 0 (ET_0/RC), the efficiency with which a trapped exciton can move an electron into the electron transport chain (ψE_0), the maximum quantum yield of primary photochemistry (ϕP_0, also known as F_v/F_m), and the quantum yield of electron transport (ϕE_0), decreases with increasing Cu concentration (*Xia and Tian 2009*). However, the initial rise in the ABS/RC, energy flux for trapping at time 0 (TR_0/RC), and ET_0/RC can be followed by a significant decline corresponding to the increasing concentration of Cu^{2+} (*Jin et al. 2021*). The photosynthetic performance index (PI_{ABS}), ϕP_0, ϕE_0, ψE_0, and driving force (DF_{ABS}) are also inhibited by Cu^{2+} in a concentration dependant manner. Besides the inactivation of reaction centres, altered fluorescence and quenching parameters under Cu stress suggest damage to the plant photoprotection mechanisms (*Możdżeń et al. 2017, Rocha et al. 2021*). An increase in the expression of proteins involved in photosynthesis, enzymes of carotenoid synthesis, and CBB cycle (*Rodríguez et al. 2018*), as well as in respiration, and the assimilation of vital nutrients, can be one of the mechanisms of tolerance of excess Cu concentrations (*Laporte et al. 2020*).

Selenium (Se)

Se generally affects several physiological and biochemical processes in plants in a dual manner. At low concentrations, Se can have beneficial effects, and at higher concentrations, it can become toxic (*Hawrylak-Nowak et al. 2015, Štolfa et al. 2017*). Most research nowadays deals primarily with the beneficial effects of Se in different types of abiotic stresses due to its role in alleviating oxidative damage in many plant species (*Feng et al. 2013, Liu et al. 2023*). Those researchers have indicated that Se treatment improves growth in many plants exposed to different environmental stressors associated with increased photosynthetic efficiency. Se treatment increased F_v/F_m, Φ_{PSII}, and nonphotochemical quenching coefficient (qN) in the salt-stressed plants, indicating that Se might decrease the reduction of photochemical conversion efficiency and photochemical activity of PSII (*Diao et al. 2014*). These results showed a protective role of Se on PSII from overexcitation through regulation of a photo-protective mechanism under salt stress, which could cause the loss of integrity in the thylakoid membranes. *Alves et al. (2020)* found that Se application increased E, g_s, and maximum area-based rate of light-saturated CO_2 assimilation compared to the control. However, Se treatment combined with Cd stress does not alleviate cadmium-stress damages in plants. Hence, plants treated with Se under Cd stress exhibited similar changes to those observed in plants exposed to Cd only. On the contrary, Se treatment restored the decline of quantum yield for reduction of end electron acceptor at the PSI acceptor side (δR_0), ϕP_0, ϕE_0, and the probability that a PSII chlorophyll molecule functions as RC (Y_{RC}) to the control level in Cd stressed leaves showed by the JIP test. Also, Se supplementation recovered ABS/RC and TR_0/RC in Cd-stressed plants (*Li et al. 2020*). Under high-temperature stress, *Djanaguiraman et al. (2010)* found that Se application increased the photosynthetic rate by 30% but, at the same time, did not affect chlorophyll content, F_v/F_m, or the ratio of ground state to maximum fluorescence (F_0/F_m). Positive Se effect is also registered under drought stress. Se pretreatment under drought stress increases relative water content, total chlorophyll content, and photosynthetic efficiency (increases in F_v/F_m and performance index (PI)). In leaves, Se enhanced NPQ, which resulted in a slight but significant decrease of the effective quantum yield of PSII in light adapted state (Y(II)). Interestingly, this increase in NPQ was coupled with non-modified cyclic electron flow around PSI, which indicates that other factors might be involved in the enhancement of NPQ. While the elevation of NPQ usually occurs in response to suboptimal environmental conditions as a defense mechanism, in leaves, Se treatment likely triggered a eustress-like response in PSII, as Se treatment affected PSII electron transfer processes (*Borbély et al. 2021*). In the salt-stressed seedlings treated with low concentrations of Se, parameters F_v (variable fluorescence), F_v/F_0, F_v/F_m, and Y(II) were increased, showing improvements in PSII photochemical activity under salt stress. However, at high Se concentrations, the damage intensified due to increased Se accumulation in the leaves (*Liang et al. 2020*). Higher concentrations of Se exhibit toxic effects in plants, mainly through the reduction of Chl biosynthesis and inhibition of the enzymatic kinetics or electron transport chain in photosynthesis (*Hasanuzzaman et al. 2020*).

Lead (Pb)

Unlike Fe and Cu, which are essential for plants, especially in photosynthesis, Pb is one of the most widespread, persistent, and phytotoxic nonessential HMs. The toxic action of Pb is seen through many effects which directly or indirectly disrupt the photosynthetic activities (*Mitra et al. 2020* and references therein). The direct impairment of photosynthesis works on multiple levels through alterations in the number and ultrastructure of chloroplasts, pigment biosynthesis, the efficiency of light reactions, the thioredoxin system, and Calvin-Benson-Bassham (CBB) cycle effectiveness, as well as the synthesis and distribution of carbohydrates. Indirectly, by impairing plants' redox state, Pb induces ROS generation, which acts *in situ* or targets different objectives outside chloroplasts (*Tokarz et al. 2020* and references therein).

The gas-exchange parameter values vary with the increasing Pb concentrations. P_N increases under 200 mg kg^{-1} added Pb, while a strong reduction is observed when the Pb concentration exceeds 200 mg L^{-1}, with a similar variation in g_s and E (*Yang et al. 2020*). Under Pb treatment, P_N decreases in both Pb-stressed and Pb-tolerant plant varieties (*Xia et al. 2019*). Despite the reduction in P_N under Pb stress, the Pb-tolerant plant varieties can adapt and increase photosynthetic performance by increasing the intracellular CO_2 concentration (C_i), g_s, and E. Increased C_i and g_s under high Pb concentrations are one of the mechanisms for maintaining the photosynthetic capacity of Pb-tolerant plants (*Pereira et al. 2014*).

Low Pb concentration (≤ 2 mM) can reduce P_N, E, and g_s without affecting the C_i, and ChlF parameters, while longer and more severe Pb treatment (10–30 d; ≥ 4 mM) inhibits P_N, which is followed by an increase in C_i and a decrease in g_s, E and ChlF parameters in longer exposures (*He et al. 2018*). A decrease is also observed for P_N, E, g_s, C_i, F_v/F_m, photochemical quenching (qP), and Φ_{PSII}, while in contrast, the minimum fluorescence (F_0) and qN showed an increasing trend (*Zhou et al. 2018*). Exposure to 40 μM $Pb(NO_3)_2$ induced a high reduction of P_N in Pb-hyperaccumulator plants due to root membrane damage, resulting in stomatal closure and decreased CO_2 availability (*Leal-Alvarado et al. 2016*). Pb interferes in the electron transport chain at both PSII and PSI levels. Pb induced a relative decrease in the ChlF induction curve, which resulted in the decrease in maximal fluorescence yield (F_m), F_v/F_m, and Φ_{PSII}, while qN, the quantum yield of regulated (Y_{NPQ}) and nonregulated energy dissipation (Y_{NO}) of PSII, increased significantly (*Kumar and Prasad 2015*). Furthermore, Pb also caused a decrease in maximal P700 change (P_m), photochemical quantum yield (Y_{PSI}), nonphotochemical quantum yield (Y_{ND}), and ETR of PSI (*Kumar and Prasad 2015*).

Cadmium (Cd)

Cd is one of the most harmful HMs due to its acute toxicity, high water solubility, and non-degradability (*Shaari et al. 2024*). Cd treatment can disturb photosynthesis at different structural and functional levels: leaf chlorophyll structure and light capture complexes, thylakoid ultrastructure and photosynthetic electron transport, stomatal conductance and access of CO_2, activities of CBB cycle enzymes, and accumulation of ROS (*Krupa 1999, Sarvajeet et al. 2011, Parmar et al. 2013, Xue et al. 2013, Alves et al. 2020*).

The contradictory effects of Cd on photosynthetic performance may originate from differences in the experimental conditions like applied Cd concentration and duration of Cd exposure, as well as characteristics of plant species (*Dias et al. 2013, Xue et al. 2014, Paunov et al. 2018*).

Alves et al. (2020) noticed the negative effect of Cd on the ultrastructure of chloroplasts that involved thylakoid disorganization, dilated thylakoid membranes, and an increase in the size and number of plastoglobuli and starch grains. These changes in chloroplast ultrastructure could indicate metabolic dysfunction, leading to a reduction in the photosynthetic rate.

PSII is generally more sensitive to Cd than PSI (*Mallick and Mohn 2003, Wang et al. 2022*). Cd affects both the donor and acceptor sides of PSII. OEC is inhibited on the donor side, while electron transport between Q_A^- and Q_B^- is inhibited on the acceptor site (*Sigfridsson et al. 2004*). The presence of Cd ions also increases qN (*Janeczko et al. 2005*). *Janeczko et al. (2005)* also found decreased RC/CS, ET_0/CS, and the activity of OEC. At the same time, F_v/F_m was the least sensitive to Cd treatment. Although, there are some contradictions regarding the tolerance of PSI and PSII to Cd (*Chugh and Sawhney 1999*). In cyanobacteria, short exposure to Cd causes PSI acceptor side limitation and a generation of excitation pressure on the reaction centers. Inhibitory effects on PSII electron transport and the degradation of the reaction center protein D1 can only be observed after prolonged exposure and only in light (*Tóth et al. 2012*). *Song et al. (2019)* found that the shape of the chlorophyll *a* induction curves (OJIP) in leaves was altered under different Cd treatments. The increase in the OJ phase and the decrease in the IP phase in fluorescence induction curves suggested that Cd toxicity inhibited both light use efficiency and photodamage avoidance ability. Also, the decreased electron transport per cross-section (ET_0/CS_m) and relatively stable non-photochemical quenching per CS (DI_0/CS_m) may cause the imbalance between light energy absorption by photosystems and energy consumption by metabolic sinks in the plant. A similar increase in the OJ phase was found by *Peršić et al. (2022)* in plants treated with Cd. They also found increased amplitude of the Lband's (ChlF transient normalized between O-K steps) positive deviations with Cd concentrations and exposure time, which leads to a more significant loss of antenna connectivity due to Cd stress. *Franić et al. (2020)* found that in ear-leaves sensitive to Cd, treatment with Cd-induced an increase in dissipation energy (ϕD_0, DI_0/RC), decreased density of reaction centers (RC/ABS), and decreased contributions of light reactions and electron transport for primary photochemistry (TR_0/DI_0 and ($ET_0(TR_0–ET_0)$)), respectively, suggesting blockage of electron transport from reaction centers to quinone (re-oxidation problems of primary quinone acceptor (Q_A)).

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Part 3

Involvement of Chlorophyll *a* Fluorescence Method in Agronomy

Chlorophyll Fluorescence Imaging in Assessing Crop Abiotic Stress

Boris Lazarević

Introduction

Crops are frequently exposed to different abiotic stresses in the field. Understanding the abiotic stress responses is a prerequisite for creating stress-tolerant crops as the principal target of many researches and breeding programs. Thus, efficient and reliable tools and methods for screening for abiotic stress tolerance are required. One such technique is chlorophyll fluorescence analysis, which is a non-invasive measurement of photosystem II (PSII) activity (*Maxwell and Johnson 2000, Murchie and Lawson 2013*). The sensitivity of PSII activity to abiotic factors has made chlorophyll fluorescence analysis an essential technique for assessing plant responses to its environment (*Maxwell and Johnson 2000, Murchie and Lawson 2013*), and thus for the assessment of crop tolerance to abiotic stress. Research interests of the Plant Ecophysiology group from the University of Zagreb, Faculty of Agriculture (FAZ) span from the basic understanding of the abiotic stresses in crops, effects of the abiotic stress on the crop performance in the field, enhanced nutrient acquisition by the crop plants, and the plant phenotyping as a basis for the development of tolerant crop genotypes. Because plant reactions to abiotic stresses are multisystemic and different plant organs and parts show spatial variability in the responses to abiotic stress (*Lazarević et al. 2022a*), to answer our research questions we are using a combination of techniques such as gas exchange analysis, multispectral imaging, 3D multispectral scanning, and the whole plant chlorophyll fluorescence imaging, which represents a cornerstone of our research.

Our main research activities are conducted within the Plant Phenotyping Laboratory which was formed within the framework of the Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv). Establishing end equipping of Plant Phenotyping Laboratory at the CoE CroP-BioDiv and FAZ allowed us to participate as a member country of the European Infrastructure for Plant Phenotyping Network (EMPHASIS).

This chapter tries to summarize the information about the chlorophyll fluorescence imaging technique and show examples of the application of this method in crop ecophysiology-focused research.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence analysis has been one of the most powerful and widely used techniques in plant ecophysiology research for a long time (*Maxwell and Johnson 2000*). However, the development of chlorophyll fluorescence imaging expanded the possibility of its application and made it one of the most essential high-throughput plant phenotyping techniques. In the last decades, the unraveling and understanding of crop genomes has made tremendous progress thanks to continually expanding genomic technologies. However, due to a lack of high-quality phenotypic data, the impact of genomic data on crop improvement remains unsatisfactory (*Tuberosa 2012*). Thus, the development and utilization of high-throughput phenotyping have remained a bottleneck within enhanced breeding programs and marker-assisted selection. Recent developments in imaging systems, automation of image acquisition, and development of tailored software which can extract meaningful data (phenotypic traits) from the collected images have enabled rapid progress in plant phenotyping. These advances are precious in plant phenotyping research. For some excellent recent reviews about high-throughput phenotyping, please see *Humplík et al. (2015)*, *Furbank et al. (2019)*, *van Eeuwijk et al. (2019)*, *Zhao et al. (2019)*, *Yang et al. (2020)*, *Singh et al. (2021)*. Thus, one of the most valuable innovations in chlorophyll fluorescence technology has been the development of chlorophyll fluorescence imaging, which includes advances in light emission technology, imaging detectors, and rapid data processing (*Gorbe and Calatayud 2012, Brestic and Zivcak 2013*).

In addition to increasing throughput and automating the analysis, fluorescence imaging solves other limitations of traditional point-based chlorophyll fluorescence measurements, such as heterogeneity of photosynthesis across the leaf or even the entire plant (**Figure 1**) (*Baker 2008, Bresson et al. 2015*). Fluorescence imaging can be used at the microscopic level (*Oxborough and Baker 1997*), at the leaf or plant level (*Calatayud et al. 2006*), or for remote sensing (*Calatayud et al. 2006, Gorbe and Calatayud 2012*). However, due to the complexity of plant responses to its environment and the heterogeneity of the chlorophyll fluorescence among and within plant organs, for agricultural-focused research, the most important systems are those which can capture images of the whole plant (**Figure 1**). During the past decades, several chlorophyll fluorescence imaging systems have been developed as stand-alone devices such as the IMAGING-PAM M-Series (Heinz Walz, Germany), or often automated to a certain extent and combined with other imaging techniques (RGB, multispectral, hyperspectral, thermal, hyperspectral), such as CropReporter and CropExplorer (PhenoVation, Netherlands), PlantScreen (Photon System Instruments, Czech Republic), PhenoAIxpert and Scanalyzer (Lemnatec, Germany). At our research facility, we have been equipped with the CropReporter since 2019 (PhenoVation B.V., Wageningen, Netherlands).

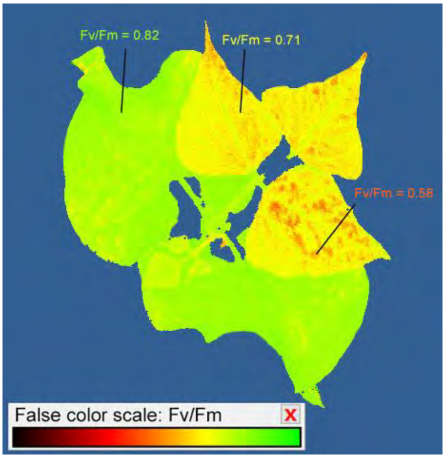

Figure 1. Example of the false color image of the maximum efficiency of PSII (F_v/F_m) in common bean (*Phaseolus vulgaris*) plant. The image was acquired by the CropReporter™. The spatial heterogeneity of the F_v/F_m among different plant/leaf parts (caused by iron deficiency) is shown, and three exact F_v/F_m values at the pixel level are shown (source: Lazarevic B, 2023).

Description of the chlorophyll fluorescence imaging protocol

For the chlorophyll fluorescence imaging, we are using CropReporter™ (PhenoVation B.V., Wageningen, Netherlands) (**Figure 2**). CropReporter™ consists of a cabinet with a camera system that houses a controller computer, a charge-coupled device (CCD) camera with an optical filter wheel and a focusing unit, integrated high-intensity red light-emitting diodes (LEDs) for excitation of the photosynthesis, LEDs at six spectral bands [broadband white (3000 K), far-red (730 nm), red (660 nm), green (520 nm), blue (460 nm), and UV/blue (405 nm)], controllable in intensity (0–780 mmol m^{-2} s^{-1}), and a spectrum for spectral imaging. All images are captured with the same lens (10 Mp lens, 200 Lp mm^{-1} resolution, 400–1000 nm spectral range) and CCD camera (1.3 Mp, 1296 × 966 pixels), with real 14-bit signal resolution. Plants can be imaged from a 30–100 cm distance from the camera. For the imaging process, there are pre-defined protocols, but new customized protocols can be created. Protocols define the imaging conditions such as focusing, binning, light adaptation period, actinic light intensity, saturation light intensity, number of frames captured for the induction curve, integration time for capturing the chlorophyll fluorescence images etc.

Figure 2. Image of the CropReporter with two plants placed inside the imaging chamber (source: Lazarevic B, 2023).

An example of a commonly used imaging protocol for the whole-plant chlorophyll fluorescence imaging will be explained in the following few sentences. Plants are imaged from a 70–80 cm distance. Prior to measurements, plants are dark-adapted for a minimum of 30 minutes. For the excitation of photosynthesis, 4000 μmol m^{-2} s^{-1} red LED light is used. The integration time for capturing the chlorophyll fluorescence image is 200 μs. The minimum chlorophyll fluorescence (F_0) and maximum chlorophyll fluorescence (F_m) images are captured after ten (10) μs and 800 ms, respectively. After the measurement, plants are relaxed in the dark for 15 s and then adapted to the light using actinic light of 300 μmol m^{-2} s^{-1} for 5 minutes. Steady-state fluorescence yield (F_s') is measured before the onset of the saturating pulse, and maximum chlorophyll fluorescence (F_m') of light-adapted leaves is measured at saturation, using the saturating pulse intensity (4000 μmol m^{-2} s^{-1}). After the measurement, actinic light is turned off, and in the presence of far-red light, the minimal fluorescence yield of the illuminated plant (F_0') is estimated. All measured and calculated chlorophyll fluorescence parameters are shown in **Table 1**.

Table 1. List of analyzed chlorophyll fluorescence traits with abbreviations (Abrrev), the equation for calculation, and the reference if appropriate.

Abbrev	Trait	Equation
F_0	Minimum fluorescence of dark-adapted leaves	see the description of the measurement protocol
F_m	Maximum fluorescence of dark-adapted leaves	see the description of the measurement protocol
F_s'	Steady-state fluorescence yield	see the description of the measurement protocol
F_m'	Maximum chlorophyll fluorescence of light-adapted leaves	see the description of the measurement protocol
F_o'	Minimum fluorescence yield of illuminated plant	see the description of the measurement protocol
F_v/F_m	Maximum efficiency of PSII	$F_v/F_m = (F_m - F_0)/F_m$ (*Kitajima and Butler 1975*)
F_q'/F_m'	Effective quantum yield of PSII	$F_q'/F_m' = (F_m' - F_s')/F_m'$ (*Genty et al. 1989*)
ETR	Electron transport rate	$ETR = F_q'/F_m' \times PPFD \times (0.5)$ (*Genty et al. 1989*)
NPQ	Non-photochemical quenching	$NPQ = (F_m - F_m')/F_m'$ (*Bilger and Björkman 1990*)
qP	Coefficient of photochemical quenching	$qP = (F_m' - F_s)/F_v$ (*Schreiber et al. 1986*)
qN	Coefficient of non-photochemical quenching	$qN = 1 - (F_m' - F_0')/(F_m - F_0)$ (*Schreiber et al. 1986*)
qL	Estimation of 'open' reaction centers on the basis of a lake model	$qL = ((F_m' - F_s') \times F_0'))/((F_m' - F_0') \times F_s'))$ (*Kramer et al. 2004*)
(ϕnq)	Quantum yield of non-regulated non-photochemical energy loss in PSII	$\phi nq = 1/(NPQ + 1 + qL(F_m/F_0 - 1))$ (*Genty et al. 1996*)
(ϕnpq)	Quantum yield of regulated non-photochemical energy loss in PSII	$\phi npq = 1 - \phi psII - 1/(NPQ + 1 + qL(F_m/F_0 - 1))$ (*Genty et al. 1996*)

The output is a 16-bit RAW format, and automatic analysis of chlorophyll fluorescence, color, and multispectral images on the pixel basis or averaged on the whole image basis are performed by DA™ software (PhenoVation B.V., Wageningen, Netherlands) (**Figure 3**).

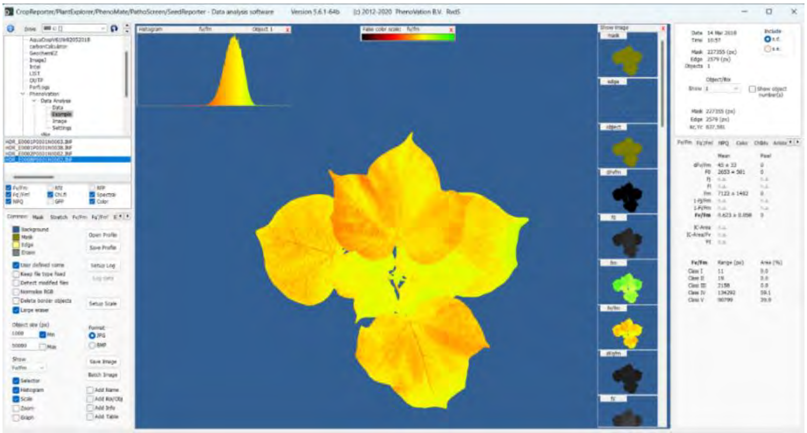

Figure 3. Example of the maximum efficiency of PSII (F_v/F_m) image and the overview of the features in DA software (source: Lazarevic B, 2023).

False color palettes are used to represent the measurements, where different colors represent different numerical values of the parameters. This type of data representation helps to show the heterogeneity of chlorophyll fluorescence in plant samples. The displayed images are like a topological map showing the values of the measured parameters of the sample (**Figures 1** and **2**).

Application of chlorophyll fluorescence imaging in plant abiotic stress

Feeding a world population of almost 10 billion people in 2050 would require raising the overall food production by more than 54 percent from the base year of 2012 (*FAO 2018*). To achieve sufficient food production, agriculture has already caused land use and utilization changes and significantly contributed to the global greenhouse gas emissions causing climate change (*Lynch et al. 2021*). On the other hand, global climate change is increasing the occurrence and intensity of unfavourable environmental conditions subjecting crops to various abiotic stresses (*IPCC 2014*). Thus, future agriculture management practices would need to increase productivity, especially on less fertile croplands, and under increased frequency and duration of different abiotic stresses, increase resource efficiency, and simultaneously decrease its effect on climate by reducing inputs such as fuel, pesticides, and fertilizers (*FAO 2018*). To be able to fulfill these requests, understanding of crop performance under abiotic stresses is needed, and this is one of the major research topics within the Plant Ecophysiology group from the University of Zagreb, Faculty of Agriculture. Chlorophyll fluorescence imaging enables spatial and temporal quantification of the effects of abiotic stress on crop photochemistry, and as such, it gives insight into the plant's physiological responses to its environment.

For example, chlorophyll fluorescence imaging was used to screen for drought tolerance among eight novel *Miscanthus sinensis* hybrids (*Lazarević et al. 2022b*). Drought treatment (21 days) reduced the maximum efficiency of PSII (F_v/F_m) from 0.79 (in control) to 0.69 in the less drought-susceptible hybrids (GRC4 and GRC5). Whereas in drought-sensitive hybrids (GRC2 and GRC3), F_v/F_m dropped from 0.79 (in control) to 0.35 (**Figure 4**). The more drought-resilient hybrids showed lower phenotypic plasticity than their more sensitive counterparts and a stay-green strategy.

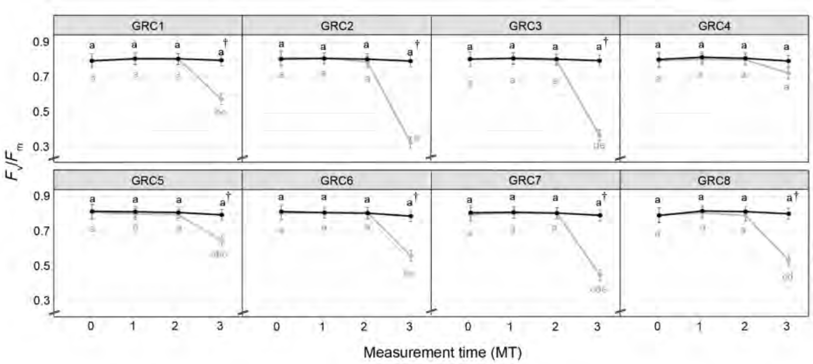

Figure 4. The maximum quantum yield of PSII (F_v/F_m) means and standard error of the mean measured at four measurement times (0 (MT0), 7 (MT1), 14 (MT2), and 21 (MT3) days after the onset of treatments) on eight *M. sinensis* hybrids (GRC1-GRC8) grown in control (black symbols) and drought treatment (grey symbols). Lowercase letters indicate significant differences among hybrids at each measurement time for each treatment (black: control, grey: drought), and the dagger indicates the measurement time at which significant differences between treatments for each hybrid occurred (Tukey's HSD test) (source: *Lazarević et al. 2022b*).

In addition, chlorophyll fluorescence imaging was used to study temperature-induced changes among three potato (*Solanum tuberosum*) cultivars (Agria, Bellarosa and Desiree). Plants were grown under increasing temperatures (thermoperiods): 20/15 °C (T1), 25/20 °C (T2), 30/25 °C (T3) and 35/30 °C (T4) for ten days. Increasing temperatures increased the minimal, steady state, and maximum fluorescence of both dark and light-adapted plants. However, a higher increase was found for F_0 and F_s' compared to F_m and F_m', which caused a decrease in F_v/F_m and F_q'/F_m', although only at higher temperature treatments (**Figure 5**) (*Lazarević et al. 2022c*).

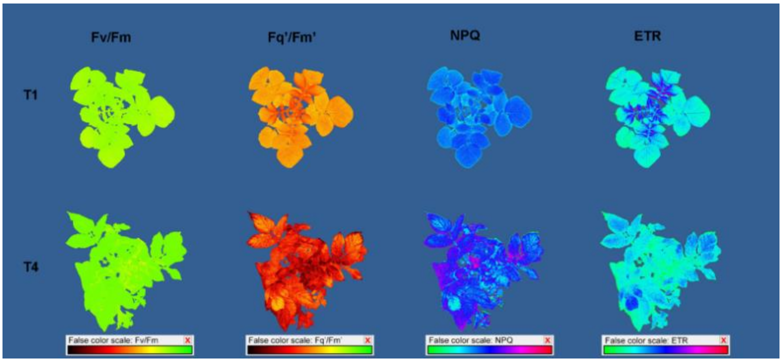

Figure 5. Potato (*Solanum tuberosum* L.) pseudo-colour images of the maximum quantum yield of PSII (F_v/F_m), the effective quantum yield of PSII (F_q'/F_m'), non-photochemical quenching (NPQ) and electron transport rate (ETR) captured by CropReporter after ten days of growth in different temperature treatments: 20/15 °C (T1) and 35/30 °C (T4). Light adaptation was performed using 250 µmol m^{-2} s^{-1} (source: *Lazarević et al. 2022c*).

Chlorophyll fluorescence imaging and multispectral imaging were used to discriminate between drought and salinity stress in basil (*Ocimum basilicum*) (*Lazarević et al. 2021*). Both drought and salinity are osmotic stresses, and are often difficult to distinguish from each other. Chlorophyll fluorescence imaging revealed that both drought and salinity stress had a more substantial effect on NPQ, ETR and F_q'/F_m' compared to F_v/F_m, which was affected only by severe drought (**Figure 6**). Previous studies have shown that F_v/F_m is not sensitive to early or moderate water stress (*Massaci et al. 2008*) or salinity stress (*Baker and Rosenqvist 2004, Awlia et al. 2016*). On the other hand, the observed increase in NPQ and a concomitant decrease in F_q'/F_m' and ETR are in line with previous reports on drought (*Yao et al. 2018*) and salinity (*Awlia et al. 2016*). Besides, under prolonged stress, the NPQ is higher and ETR is lower in severe salinity than in drought stress, indicating the ion toxicity caused by prolonged exposition to salinity stress. Electron transport rate (ETR) (along with anthocyanin index) was found as the best indicator for differentiation of salinity-stressed plants from non-stressed plants both in early and prolonged stress (*Lazarević et al. 2021*).

Similarly, discriminant analysis was used to compare the efficiency of the chlorophyll fluorescence traits in distinguishing between early and prolonged nutrient deficiency (nitrogen (N), phosphorus (P), potassium (K), magnesium (Mg), and iron (Fe)) in common bean (*Phaseolus vulgaris*) (*Lazarević et al. 2022a*). Furthermore, a recursive partitioning analysis (*Therneau and Atkinson 2022*) was used to select chlorophyll fluorescence traits that show the highest accuracy for assigning plants to the respective nutrient deficiency treatments. Plants were grown in treatment solutions (control and solutions lacking one of the nutrients: N, P, K, Mg, and Fe), and imaging was performed 3 (MT1), 6 (MT2), 9 (MT3), and 12 (MT4) days after the onset of treatments. Discriminant analysis showed that classification success for separation among nutrient deficiency treatments (Control, N, P, K, Mg, and Fe) using the entire set of chlorophyll fluorescence traits achieved 80% at first measurement time (MT1) and 98.3% at subsequent measurements. However, the recursive partitioning model MT4 selected F_q'/F_m', F_v/F_m, NPQ, and F_m as traits that were best for assigning plants into their respective nutrient deficiency groups, resulting in 81.7% of accuracy. Selected chlorophyll fluorescence traits showed high accuracy for assigning plants into control, Fe, Mg, and P deficit, but could not correctly assign K and N deficit plants (**Figure 7**).

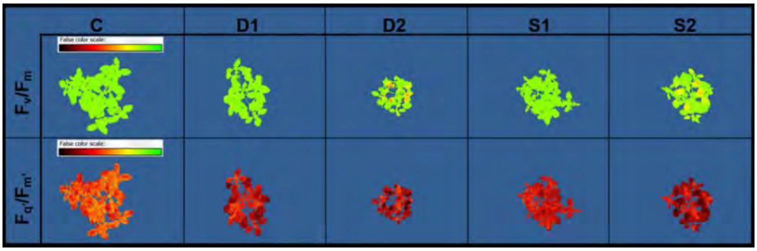

Figure 6. Basil pseudo-color images maximum quantum yield of PSII (Fv/Fm), and the effective quantum yield of PSII (Fq'/Fm') under control (C), moderate drought stress (D1), severe drought stress (D2) moderate salinity stress (S1), severe salinity stress (S2), captured by CropReporter at the second measurement time (14 days after onset of treatments). Fq'/Fm' image was captured at 250 µmol m^{-2} s^{-1} (source: adapted from *Lazarević et al. 2021*).

Chlorophyll fluorescence imaging is a reliable, nondestructive tool that is ideally suited for plant eco-physiological research to monitor and quantify abiotic stress responses at the leaf or whole-plant level. This methodology solves the problem of sample heterogeneity and avoids potential errors that can occur with point measurements. In addition, the integration of chlorophyll fluorescence imaging systems into the plant phenotyping platforms enabled automatic measurements of chlorophyll fluorescence traits on a large number of samples. This has increased the applicability in screening for desirable agronomic traits in various breeding programs. Also, the simultaneous combination of chlorophyll fluorescence measurements with other phenotyping techniques such as multispectral imaging, thermal imaging, 3D scanning, etc., offers new possibilities in plant research and enables detailed study of the temporal and spatial interaction between plant and environment.

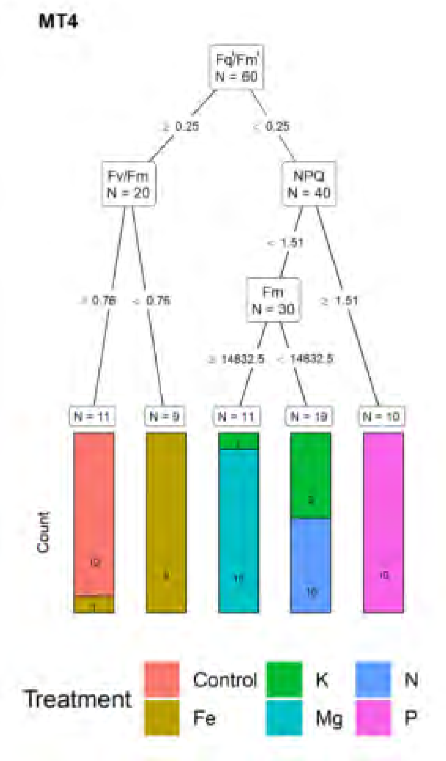

Figure 7. Visualization of classification tree for chlorophyll fluorescence traits. Each node shows the variable chosen as the best for the split in the data and the number of observations at that node (N). On the edges, between nodes, are threshold values of the split variables. Bar charts at each terminal node (leaf) represent the number of observations classified into each treatment (indicated by a different color). MT4 represent measurement time (12 days after the onset of nutrient deficiency treatments) (source: adapted from *Lazarević et al. 2022a*).

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Wheat Breeding Efforts Toward Improvement of Grain Yield Related to the New Challenges for Photosynthesis Research

**Valentina Španić, Katarina Šunić, Jurica Duvnjak,
Marija Viljevac Vuletić and Zorana Katanić**

Wheat (*Triticum aestivum* L.) is the third most-produced cereal after rice and maize, with alignment in staple crops for an estimated 35% of the world population. Currently available data for wheat indicate an average annual global production of about 780 million tons (*FAO 2022*). However, demand for wheat will increase by 50% by 2050 due to the human population increasing up to 9 billion people or more (*World Bank 2022*). Therefore, feeding the population in the future is one of the world's biggest challenges. Furthermore, unfavourable climate conditions and limited availability of arable land will reduce wheat grain yield and end-use quality (*Barlow et al. 2015*). The increase in global food insecurity has already started, in large part due to climate change that contributed to increased abiotic stress which encompasses higher temperatures, water scarcity, droughts, floods, and greater CO_2 concentrations in the atmosphere (*Lobell et al. 2015, Farooq et al. 2022*). In addition to the growth of population and rising abiotic stresses, biotic stresses pose a great threat to wheat production over the globe due to climatic changes resulting in the geographical movement of pathogens or pests (*Launay et al. 2014, Gullino et al. 2022*). Thus, the production of wheat crops varies by country and region where fungal diseases such as Fusarium head blight (*Fusarium* spp.), rusts (*Puccinia* spp.), powdery mildew (*Blumeria graminis* f. sp. *tritici*), Septoria leaf spot (*Septoria tritici*), Septoria glume blotch (*Septoria nodorum*) along with pests like cereal leaf beetle (*Oulema melanopus*) and orange wheat blossom midge (*Sitodiplosis mosellana*), etc. hinder the potential grain yield performance of the elite wheat varieties to a great extent. Furthermore, narrow genetic diversity in wheat at the local level has resulted in pathogen regeneration to some extent. Sometimes agricultural management options become important in situations where the frequency of pest and disease outbreaks is too infrequent and variable. Increasing climate variability and world population resulted in an influence on food demand and global food security that can be preserved by the creation and production of new wheat varieties through new biotechnological approaches, but it is also absolutely necessary to conserve the global wheat biodiversity.

Considering all those facts, agriculture is the most sensitive sector to climate effects, directly impacting the economic status of countries where, due to weather changes, elevated risk of hunger and malnutrition is expected (*del Mar Hidalgo García 2013*). Considering nutrition, wheat is very important, primarily as a source of energy (carbohydrate), but also because it provides significant amounts of other important nutrients including protein, dietary fiber, and other nutrients including lipids, vitamins, minerals, and phytochemicals which allow healthy nutrition (*Shewry and Hey 2015*). The predominant source of human dietary carbohydrates is starch which is important in the end-use quality of wheat (*Stone and Morell 2009*). Furthermore, in favor of its importance goes the fact that it constitutes about 60–70% of the mass of wheat grain. Protein content generally varies from about 10–15% of the dry weight for wheat genotypes grown under field conditions. Other constituents of wheat grains, such as vitamin B, could be found in bran or germ, but all grain compounds differentiate due to the effects of genotype and environment.

Grain yield is a quantitative trait, which is controlled by minor genes, whereas its expression is the result of genotype (G), environmental factors (E), and GE interaction (*Španić et al. 2021a*). There are two major sources of assimilates used for grain filling, i.e., the post-anthesis assimilates where the main source is the flag leaf, and the pre-anthesis assimilates stored in the stem. Under optimal conditions, post-anthesis photosynthesis contributes to greater grain yield (*Chang et al. 2020*). It was reported that an increase in wheat grain yield could be achieved by selection for stay-green traits (*Gregersen et al. 2013*). That means that plants may maintain greener leaves and photosynthesis capacity around anthesis before the onset of senescence or senesce more slowly (*Kamal et al. 2019*). *Christopher et al. (2016)* concluded that selection for particular stay-green traits and/or molecular markers associated with these traits could enhance genetic progress toward stay-green genotypes with higher, more stable grain yield in different environments. Thus, this can contribute to longer grain-filling, resulting in increased grain yield. Therefore, we detected that there is a clear need to improve our understanding of the genetic architecture of resistance to biotic and abiotic stresses by searching for the efficient utilization of wheat genotypes with prolonged photosynthesis activities. Nevertheless, the increase in grain yield and quality of the wheat genotypes at Agricultural Institute Osijek has been an ongoing task of wheat breeders for many years. Up to now, advances in wheat breeding and biology, genetics, and agricultural practices have been the initiators for the accelerated increase in grain yields or improvement of end-use quality. Also, the measurement of wheat photosynthesis as a relatively new biotechnological approach for quantifying photosynthetic efficiency has the role of speeding up the process of development of high-yielding wheat varieties in order to enhance even greater food production. Approximately ten research reports based on photosynthesis activity have been published or presented so far within our research group under wheat breeding activities or scientific projects.

Our first investigation related to photosynthetic efficiency in winter wheat started during the 2011/2012 growing season in order to bring out biophysical parameters of photosystem II (PSII) functioning calculated by JIP test. It is known that in the field conditions, photosynthetic parameters can be evaluated using various techniques without causing damage to plants by using visual assessments and different devices such as SPAD meter for chlorophyll measurement (*Ghosh et al. 2020*), normalized difference vegetative index (NDVI) meter (*Kizilgeci et al. 2021*), and plant efficiency analyser for chlorophyll *a* fluorescence (*Botyanszka et al. 2020*). Furthermore, high-throughput sensor-based phenotyping with advanced image analysis is able to detect and quantify the tissue substances such as the content of chlorophyll and anthocyanin (*Gitelson et al. 2002*). Thus, multispectral, hyperspectral, fluorescence and laser sensors and cameras can detect changes in biochemical, water content, biomass, morphological, phenological, yield- and root-related traits (*Thoday-Kennedy et al. 2022*) by reflectance values detected at different wavelengths. One of the examples is the sensor (OCTOflux) which allowed the increase of the phenotyping capabilities (*Salter et al. 2018*). For gas exchange measurements and chlorophyll *a* fluorescence device such as LI-COR portable photosynthesis system can be used (*Nazeer and Paulsen 2005*). Thus, photosynthetic processes are also measured at the cellular level (*Murchie et al. 2018*).

During an eleven-year investigation related to wheat photosynthesis activities at Agricultural Institute Osijek, we were using a Plant Efficiency Analyzer (HandyPEA, Hansatech, UK) for chlorophyll *a* fluorescence measurement. The usage of this device is justified by the fact that chlorophyll *a* fluorescence is one of the most promising tools used in assessing photosynthetic efficiency and provides valuable information that is relevant to plant health and wheat management (*Liu et al. 2019*). The shortcoming of our first research was the number of wheat genotypes studied, as well as the timing of chlorophyll *a* fluorescence measurement that was carried out only once prior to the harvest. It was detected that investigated wheat genotypes had significantly different photosynthetic efficiency but significant correlations between grain yield and photosynthetic parameters were not found (*Španić et al. 2013*), although not all chlorophyll *a* fluorescence parameters were taken into account which may have led us down the wrong path. Taking shortcomings into account, the results from our first investigation could not be taken as a valid conclusion. This is supported by the fact that traits such as the onset of leaf senescence vary between genotypes, and the date of measurements can significantly impact the results (*Christopher et al. 2014*). Based on our first measurements, we wanted to further explore whether conversion efficiency can be improved in the breeding process, taking into account the presence of existing genetic variation in wheat germplasm as the main prerequisite. According to *Robles-Zazueta et al. (2022),* radiation use efficiency (RUE; dry weight biomass produced per unit radiation intercepted) through the enhancement of photosynthesis gives a great opportunity to improve grain yield.

Under the scientific project of the Croatian science foundation (2015-2018) titled "Wheat breeding for resistance to Fusarium head blight combined with mycotoxin analysis and antioxidant pathways", the investigation aim related to photosynthesis was to determine the potential of recently described stay-green traits to improve knowledge about wheat adaptation to Fusarium head blight (FHB) influence. Shortly, FHB is a dangerous fungal wheat disease that can damage grains, resulting in the loss of grain yield and quality. *Fusarium* infection is favored by humid and warm conditions during wheat flowering while FHB symptoms occur a few days after infection that could be seen as a bleaching point on spikelets and further spread over the entire spike or on just a few spikelets. As high production loss during the epidemic years may appear, we wanted to explore all possible biotechnological approaches to investigate *Fusarium* pathogen-plant interactions and to find a way to combat FHB. Therefore, in this project, we incorporated chlorophyll *a* fluorescence measurement on the flag leaves of wheat genotypes in field conditions with and without *Fusarium* infestation. During the 13th International Wheat Genetics Symposium in Tulln, Austria, at a poster session, we presented our first results where our preliminary work about chlorophyll *a* fluorescence was revealed. In this study, we provoked epidemic FHB conditions to predict reactions of wheat genotypes' FHB resistance or susceptibility. Simultaneously with FHB disease assessment, we measured chlorophyll *a* fluorescence of the flag leaves after *Fusarium* inoculation on a couple of occasions prior to maturity. We chose to measure photosynthetic activity on the flag leaves because it is known that the increases in leaf photosynthesis generate a remarkably enhanced production of different carbohydrates (*Ainsworth and Rogers 2007*) and that the contribution rate of flag leaves to daily photosynthetic products varies from 50 to 60% (*Towfiq et al. 2015*). Furthermore, flag leaves are the organs that determine the grain-filling rate and the final grain yield (*Vicente et al. 2018*). During the grain-filling stage, biotic and abiotic stresses stimulate the accumulation of reactive oxygen species (ROS), whereas the reduction in the accumulation of assimilates in wheat flag leaves will occur (*Christopher et al. 2014, Hasanuzzaman et al. 2020*). Our results showed that the more FHB resistant genotype was less affected by FHB infection in terms of photosynthetic function in flag leaves, although the plant leaves are not primary sites of FHB infection.

This was indicated by a small reduction in performance index (PI) and maximum quantum yield of PS II (TR_0/ABS), accompanied by a lesser reduction in grain yield and test weight under *Fusarium* infection, compared to more FHB susceptible genotype (*Španić et al. 2017a*). Previous research already concluded that improving photosynthetic CO_2 assimilation is a goal for increasing wheat productivity (*Parry et al. 2011*). Furthermore, we concluded that more FHB susceptible wheat genotypes could accelerate senescence in the presence of FHB stress compared to resistant FHB genotypes. This is in accordance with the conclusion that senescence accelerates in the presence of biotic or abiotic stresses (*Joshi et al. 2019*).

Besides chlorophyll *a* fluorescence influence on grain yield and yield-related traits, we wanted to investigate the effects of the chlorophyll *a* fluorescence parameter on protein and gluten components that are responsible for the end-use quality of wheat. In the research of *Španić et al. (2017b)* chlorophyll *a* fluorescence transient at 1, 2, 4, 7, and 14 days after *Fusarium* inoculation and post-harvest traits related to end-use quality were measured. Our results indicated that the maximum quantum yield of photochemistry (TR_0/ABS) and the performance index (PI) were not significantly affected by FHB in the flag leaves, but there were significant differences in those two parameters between different genotypes and measurement times. Thus, existing genotypic variation in photosynthetic efficiency can be exploited by identifying promising genotypes and traits for implantation into breeding programs (*Lawson et al. 2012*). In the research of *Španić et al. (2017b)* FHB caused a significant reduction in the percentage of glutenins (GLU), high-molecular-weight (HMW), and low-molecular-weight (LMW) subunits in moderately FHB resistant and susceptible genotypes, while in resistant genotypes, the percentage of GLU increased. The gluten complex, which determines the technological wheat quality, is consisted of gliadins and glutenins. Furthermore, glutenins are divided into HMW and LMW glutenins. The formed proteins are transferred to the grain after wheat deflowering (*Dupont and Altenbach 2003*) and therefore, in our research, were significantly influenced by FHB, although main chlorophyll *a* fluorescence parameters were not impacted. We can make a hypothesis that a sink rather than a source limitation occurred during grain filling in FHB susceptible genotypes. According to *Sultana et al. (2021)*, early senescence is related to high grain protein content with low yield, while late senescence is often related to high yield and lower protein content.

Furthermore, *Viljevac Vuletić et al. (2019)* went deeper into research related to photosynthesis by evaluating photosynthetic (JIP test) and oxidative pathways from flowering till the late milk development stage in the flag leaves of two wheat genotypes differing in grain yield. The maximum quantum yield of PSII (TR_0/ABS) and performance index on absorption basis (PI_{ABS}) indicated functional photosynthetic activity, but, according to other JIP test parameters, down-regulation of PSII occurred in flag leaves of both genotypes. In flag leaves of low-yielding genotype, photo-inhibition was revealed, resulting in significant inactivation of active reaction centers, altogether with enlargement of carotenoids content. Simultaneously, the same genotype increased catalase activity, hydrogen peroxide (H_2O_2), and malondialdehyde content in flag leaves, thus showing oxidative stress. These accompanying actions are expected as blocking of electron transport under stress is usual. Altogether changes in photosynthetic and oxidative metabolism levels in flag leaves of low-yielding genotype implied an earlier onset of senescence. This was in accordance with the research of *Joshi et al. (2019)*, who reported that early or untimely leaf senescence results in the inadequate mobilization of leaf or stem metabolites to wheat grains. In the continued research, *Viljevac Vuletić and Španić (2020)* reported about chlorophyll fluorescence measurements and photosynthetic pigment content by evaluating the onset and rate of the flag leaf senescence every 7 days, beginning at the flowering stage (0 days after flowering, DAF), until late senescence stage (35 DAF) on winter wheat field-grown genotypes with the similar maturity. During grain filling, senescence occurs naturally, including reserve reallocation from senescing tissues into grains. As photosynthesis is performed in chloroplasts, measurements of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) as primary photosynthetic pigments were included in this research that allow the plants to absorb energy from light (*Havaux 2013*). However, carotenoids are also important as they have a role in the photo-protection of chlorophyllous pigments (*Chen and Roca 2018*). Nevertheless, both chlorophylls and carotenoids are photosynthetic pigments capable of absorbing light, transmitting energy, and accumulating chemical energy as sugars (*Racz et al. 2022*). Therefore, the size of the green leaf area, chlorophyll content, and net photosynthetic rate could have a role in forming the grain yield. In the research of *Viljevac Vuletić and Španić (2020),* the earliest symptoms of senescence onset were indicated by positive L band at 7 DAF and K band at 14 DAF. Further, the earliest indicators of senescence in wheat flag leaves were the quantum yield of energy dissipation and dissipated energy flux per reaction center. In general, the decrease in photosynthetic rate during senescence is expected due to the ultrastructural alterations of chloroplasts (*Kong et al. 2010*). The final conclusion of our work was that selection for functional stay-green traits could contribute to increasing the wheat yields.

Similar research was publicized by *Španić et al. (2020)*, showing that high-yielding genotype increased catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPOD), and polyphenol oxidase (PPO) activities earlier during the course time experiment, which could be the reason of delayed senescence process. That was expected as plants respond to oxidative stress by the induction of a defensive system including enzymatic antioxidants [superoxide dismutase (SOD); CAT; peroxidase (POD); APX] and non-enzymatic antioxidants (*Riaz et al. 2022*). Overall, genotypes having higher activity of some antioxidants are expected to produce more yield under stress conditions (*Luna et al. 2005*). Oxidative damage resulting from increased H_2O_2 accumulation with increased lipid peroxidation and a decline in antioxidative enzymes activity may contribute to accelerated senescence in a low-yielding genotype that is accompanied by chlorophyll and carotenoid decrease (*Španić et al. 2020*), as chloroplasts and mitochondria are the two main sites for the generation of ROS. Furthermore, delay in senescence in the high-yielding genotype was associated with a decrease in grain protein content.

At the end of 2019, we started to carry out a new scientific project: "Response of winter wheat to biotic and abiotic stresses caused by climate change" (2019-2022), financed by the European regional fund. We extended our previous investigation on chlorophyll *a* fluorescence of wheat to simultaneous measurements in the field and in the controlled conditions (greenhouse). Also, we started measurement on the wheat spikes beside flag leaves. A number of studies have revealed the important role of spikes under different control and stressful growth conditions (*Sanchez-Bragado et al. 2020*). Spikes can be very important in terms of photosynthate production as they are located at the upper part of the plant and thus have more light, they are closer to grains for transport of assimilates, their surface is large, and senesce later than flag leaves (*Chang et al. 2020*). Furthermore, our investigation related to photosynthesis research was expanded to experiments on drought stress, thus encompassing both biotic and abiotic stresses' influence on chlorophyll *a* fluorescence in wheat.

At the 56th Croatian & 16th International Symposium on Agriculture (Vodice, Croatia), *Katanić et al. (2021a)* presented preliminary research of this project, where a decrease in performance index (PI_{ABS}) and maximum quantum yield of PS II (TR_0/ABS) in the FHB inoculated spikes, in comparison to control (natural infection) of FHB susceptible genotype was detected three days post inoculation, indicating that changes in photosynthetic apparatus of susceptible genotype occurred before visual symptoms of the infection. Upon symptoms development, inoculated spikes showed an increase of PI_{ABS} and TR_0/ABS, while the progression of the disease resulted in reduced photosynthetic performance, in comparison to control. A less severe impact of FHB on the photosynthetic efficiency of the spikes of FHB resistant genotype was evident. Overall, this study indicated that *Fusarium* spp. infection induced changes in photosynthetic performance and functioning of PSII in wheat spikes, primary sites of infection. This statement is supported by the review of *Yang and Luo (2021),* where it was concluded that pathogen infection inevitably leads to changes in photosynthesis parameters, enzymes such as SOD and CAT, signaling molecules such as H_2O_2 and hormones, as well as expression of genes involved in photosynthesis.

In the research of *Španić et al. (2021b),* the evaluation of the maximum quantum yield of photosystem II (PSII) (TR_0/ABS) and performance index on absorption basis (PI_{ABS}) of flag leaves and glumes of heads (spikes) at the flowering stage until the mid-senescence stage was expended to two different locations in Croatia. The grain yield of all genotypes on average was significantly higher at one location, compared to the other one, but some genotypes exhibited higher yield reduction at investigated locations. This was expected as grain yield is under the great influence of the environment, thus pointing out the importance of analysis of the genotype x environment interaction (GEI) (*Subira et al. 2015*). A higher amount of precipitation in June at one location provoked higher FHB disease intensity, which could be seen as the bleaching of plant's spikes and resulted in an earlier decrease in photosynthetic activity. Therefore, earlier senescence and contracted grain fill duration could occur (*Španić et al. 2021b*). It was already stated that the difference in the degree of photosynthetic changes in the early stages of infection could, conversely, be an indicator of the resistance level in genotypes (*Yang and Luo 2021*).

We were invited to give a talk at The 2nd International Symposium of Silk Road Wheat Innovation Alliance (Yangling, China), where we partially incorporated results with chlorophyll *a* fluorescence, obtained up to now. Basically, we concluded that all tested wheat advanced lines have a good genetic potential for grain yield and showed good photosynthesis activity till 22 days after flowering when senescence started (*Španić et al. 2021c*).

Furthermore, in the published research of *Katanić et al. (2021b)*, obtained results indicated that the response of wheat to FHB includes changes in photosynthetic efficiency which can cover alternating modifications in both flag leaves and spikes. The most significant factor in modulating the response of wheat to FHB was the location, although genotype also had a significant influence. L band appearance was of particular interest, showing a similar response in all FHB inoculated plants, regardless of genotype or location. Also, the changes in the L band and K band helped with the prediction of tolerance of wheat genotypes in abiotic stress (*Jain et al. 2018*). Related to the research of *Katanić et al. (2021b)*, at the conference Power of Fungi and Mycotoxins in the Midst of Climate Change (Koprivnica, Croatia), *Španić et al. (2022a)* presented a poster where it was shown that the energy fluxes ratios absorption per active reaction center (ABS/RC), trapping per active reaction center (TR_0/RC), electron transport per active reaction center (ET_0/RC), dissipation per active reaction center (DI_0/RC) increased in the effect of dual-stress that was seen as increased accumulation of *Fusarium* and *Alternaria* metabolites in the grains, thus reducing grain yield. Opposite to that, wheat samples with less accumulation of those metabolites in grains at another experimental site kept optimal grain size through maintenance of grain fill, and thus higher overall grain yield.

At 15 International meeting Plant breeding, nursery and seed production (Zadar, Croatia), *Katanić et al. (2022)* and *Španić et al. (2022b)* presented comprehensive obtained results for photosynthetic efficiency on different wheat genotypes/advanced lines, under different locations, and FHB treatment, influencing agronomical-related traits. It was agreed that those experiments can help breeders select future winter wheat genotypes with improved grain yield, but also detect FHB infection before visible symptoms.

The results from the project are still an ongoing task, as we still need to drive conclusions about photosynthesis research from controlled conditions based on FHB and drought stress. Roughly, preliminary results of the FHB experiment in the greenhouse with chlorophyll *a* fluorescence measurement on the spikes showed that there is a difference in the response of FHB susceptible and resistant genotypes in the early stages of *Fusarium* infection, which could be related to the ability of genotypes to defend against pathogens. Considering drought tolerance, it was previously reported that several cell building materials like carbohydrates, lipids, proteins, and nucleic acid are impaired by ROS in drought stress (*Ahmad et al. 2018*), where photosynthesis and respiration are disturbed. It is known that limiting CO_2 availability due to decreased stomatal conductance has the main role in decreased photosynthesis under drought stress (*Grieco et al. 2020*). Thus, we expect to obtain a decrease in photosynthesis due to reduced activity of some Calvin cycle enzymes, inhibition of photosynthetic electron transport, and impaired photophosphorylation capacity that was previously characterized in drought stress (*Inoue et al. 2004*). The decrease of the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) corresponded with a decline in photosynthetic capacity in wheat grown in high temperatures and drought (*Perdomo et al. 2017*). Thus, plants with no limitations in terms of Rubisco content and ribulose-1,5-bisphosphate (RuBP) regeneration will be less likely to have restricted CO_2 assimilation rates, having higher production. Our preliminary results of the experiment with different drought intensities in stem elongation and anthesis stage in controlled conditions showed that a greater impact of drought is observed in the leaves and spikes of drought sensitive genotypes, whereas greater negative impact on photosynthesis could be observed.

Under project Access to Research Infrastructures activity in the Horizon2020 Programme of the EU (EPPN2020) titled: "Analyses of the influence of leaf rust (*Puccinia triticina* Erikss.) on carbohydrate and antioxidant metabolism and phytohormones of wheat by combining multispectral imaging with physiological phenotyping" wheat plants were exposed to multispectral image acquisition, using high power LEDs positioned inside light integrating hemisphere setup. In this experiment, a sensor-based technique indicated potential pathogen-related functional disturbance of the photosynthetic apparatus in the early stages of infection by leaf rust (*Puccinia triticina* Erikss.). Leaf rust is also one of the most devastating diseases on wheat causing severe economic losses through reduction of grain yield and quality. Results from this project are still not publicized. However, similarly to the project's results, the study of *Shokat et al. (2020)* highlighted the role of antioxidant and carbohydrate-metabolic enzymes in the modulation of source-sink balance in wheat. Furthermore, according to the project's results, the sensor-based technique could be used as a rapid method for the pre-symptomatic determination of leaf rust resistance or susceptibility in wheat. It was reported that this innovative biosensor can enable the detection of yellow rust (*Puccinia striiformis* f. sp. *tritici*) spores in 72 h (*Hassan et al. 2022*). By the point when symptoms of rust are visually detected on the leaf's area, 7 to 10 days after rust infection, fungicides are generally less effective. Thus, early detection of the pathogen can help to maintain the plants in healthier condition, thus keeping green leaves and chlorophylls, the primary facilitators of photosynthesis. Generally, we can conclude that changes in photosynthesis in the early stage of pathogen infection could be a causal factor influencing resistance, also interfering with other defense mechanisms, such as antioxidative compounds. Breeding for a functional stay-green trait can contribute to the improvement of wheat grain yield and end-use quality. Still, other mechanisms related to the stay-green trait need to be revealed as this can lead to genetic improvement of wheat genotypes in challenging environmental stresses.

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Chlorophyll *a* Fluorescence as a Promising Tool for Precise Phenotyping in Conventional Soybean Breeding

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Soybean prospects in Europe

Soybean (*Glycine max* L. Merr.) is the main oilseed crop of the world (*FAOSTAT 2022*), but in Europe today, it is almost equally important as one of the key constituents in the European Commission's plant protein strategy aiming to reduce Europe's dependency on protein imported from overseas (*European Commission 2019*). To reach the self-sufficiency targets for plant-based proteins and enable crop diversification that would increase farming sustainability in the European Union, more productive European soybean varieties need to be developed. Furthermore, because of the increased adverse weather frequency over the last 20 years (*Jug et al. 2018, Pejić 2022*), continuous and intensive research is necessary to create more resilient and stable varieties as an integral part of conservation agriculture. Developing varieties less susceptible to environmental stress and more efficient at capturing light energy necessitates a thorough understanding of the photosynthetic efficiency as one of the main factors influencing grain yield.

Soybean and photosynthesis

Soybean is a C3 photosynthetic pathway plant. The C3 pathway is well adapted to environments with low atmospheric carbon dioxide and highly efficient in moderate light conditions. However, it is known to be less efficient in environments with high temperatures and high atmospheric carbon dioxide levels, and it has a lower water use efficiency compared to the C4 pathway. In other words, the rate of carbon assimilation in soybean is highly influenced by various environmental factors such as temperature, light intensity, and water availability. As environmental conditions cannot be controlled, increasing soybean productivity focuses on identifying genetic variations that can maintain photosynthesis in adverse conditions. This process can be aided by chlorophyll *a* fluorescence (ChlF) measurements. ChlF is commonly used as a tool in soybean breeding across the world to identify drought-tolerant genotypes (*Iqbal et al. 2019, Jumrani and Bhatia 2019, Basal et al. 2020, Guzzo et al. 2021, Monteoliva et al. 2021, Sakoda et al. 2022*), detect physiological responses of soybean to high-temperature stress (*Djanaguirama et al. 2011*), salt stress (*Khan 2019, Luo et al. 2021*), the effects of herbicide stress (*Jurišić 2018, Li et al. 2018,*) and Cd stress (*Liu et al. 2018*), effects of potassium deficiency (*Wang et al. 2015*), ambient ozone (*Sagan et al. 2018*), etc. The study of soybean's photosynthetic processes has provided important insights into the improvement of soybean and the optimization of production for sustainable agriculture.

ChlF as a tool in breeding drought-tolerant soybean at the AIO

At the conventional, GMO-free soybean breeding programme of the Agricultural Institute Osijek (AIO), photosynthesis has been an active area of study since the beginning of the last decade. The main goal was to use ChlF as a relatively fast and reliable phenotyping tool in breeding drought-stress tolerant soybean genotypes. Drought is already an important constraint for soybean production in Central Europe, where periods of extreme water shortage occur regularly (*Pejić 2022, Nendel et al. 2023*). It is known to cause changes in stomatal regulation (*Pirasteh-Anosheh et al. 2016*), photosynthesis inhibition (*Tang et al. 2017*), increased oxidative (*Sun et al. 2020*) and metabolic changes (*Du et al. 2020*), earlier senescence, decrease in plant height (*Wei et al. 2018*), decrease in leaf area, pod yield, plant height, 1000-seed weight, and harvest index (*Cui et al. 2019*), all of which commonly reflect on the seed yield. According to the climate model projections, the negative influence of drought is inevitably going to spread across Europe in the near future (*Beillouin et al. 2020, IPCC 2021*). Furthermore, the annual summer air temperature in Central Europe is predicted to rise by 3.6–6 °C, while precipitation is predicted to decrease by 7–20% at the end of the 21st century (*Coppola et al. 2021, Politi et al. 2022*). Since European agriculture is mainly rain-fed, with only a 6% share of irrigated area (*Rossi 2019*), drought-tolerant soybean genotypes are crucial for providing resilient crop production systems and stable yields (*Arya et al. 2021, Guzzo et al. 2021, Monteoliva et al. 2021*). Therefore, our ongoing research aims to incorporate ChlF in the breeding process as a cost-effective phenotyping method for early-generation drought-stress tolerance screening, increasing the breeding efficiency without increasing the input burden of additional selection criteria.

In the screening of soybean genotypes for drought tolerance, the general conclusion is that tolerant genotypes have a higher photosynthetic rate in adverse conditions, resulting in higher yields compared to non-tolerant genotypes (*Chen et al. 2016, Guzzo et al. 2021, Monteoliva et al. 2021*). As photosynthetic efficiency can be evaluated by ChlF, our initial investigation focused on determining which ChlF parameters were the most informative for soybean genotypes created at the AIO and tested in drought conditions (*Matoša Kočar et al. 2022*). The preliminary experiment was set up in the greenhouse in plant pots with 16 elite soybean lines with superior agronomic traits. In the first experimental year, soybeans grown in sufficient water supply conditions (80% available water holding capacity – AWC) were compared to soybeans grown in drought conditions (50% AWC) in five growth stages: 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*). The ChlF was measured when soil water content reached the permanent wilting point in the drought-stressed treatment at each of the five growth stages. As expected, drought affected the functioning of the photosynthetic apparatus, with the timing of the drought occurrence being a significant source of variation. Among all ChlF parameters, the performance index for energy conservation from exciton to the reduction of PSI end acceptors (PI_{total}) was chosen as the most sensitive in detecting the average drought-stress effect in tested genotypes across five growth stages (V2, R1, R4, R5 and R6). It is known to have a higher sensitivity to abiotic stress compared to other fluorescence parameters. In plants susceptible to abiotic stress, PI_{total} usually decreases, indicating inhibition of the photosystem II (PSII) activity and structural and/or functional damage of the PSI, i.e. decreased ability for energy conservation (*Yusuf et al. 2010, Pavlović et al. 2019, Mihaljević et al. 2021*). R1, R4, and R6 were found to be the growth stages in which soybean were the most sensitive to drought stress. To corroborate the preliminary findings, two genotypes, one with superior photosynthetic efficiency and one with the lowest among tested genotypes, were chosen for further examination in a plant pot experiment (*Markulj Kulundžić et al. 2022*). Drought was simulated at the R5–R6 growth stage. Physiological measurements and tissue samplings began on the first day of drought exposure and lasted five days, i.e. until the water content in the soil reached 50% AWC. Among all, performance index on absorption basis, i.e. the efficiency of energy conservation from absorbed photons to reduction of intersystem electron carriers (PI_{ABS}), was found to be one of the most informative ChlF parameters, able to distinguish between genotypes and treatments. PI_{ABS} is known to detect the early changes caused by stress in plants (*Kalaji et al. 2016*), with its decrease being a sign of abiotic stress susceptibility (*Bano et al. 2020, Killi et al. 2020*). It is commonly used for quantifying general plant conditions and vitality and evaluating the total photosynthetic efficiency in variable environmental conditions (*Strasser et al. 2004*). According to the ranks described by *Evans (1996*), PI_{ABS} was in a moderate positive correlation with the relative water content (RWC; r = 0.59), which is known to be one of the most indicative physiological drought stress parameters (*Dong et al. 2019, Ali and Siddiqui 2022*). Such findings indicate PI_{ABS} can be used in breeding for drought susceptibility as an indicator of stress effect instead of RWC (which is more complicated and time-consuming to measure), without losing much data. This is especially helpful when there are large segregating populations in early generations of the breeding process. Although the maximum quantum yield of PSII photochemistry (TR_0/ABS) is one of the most frequently used ChlF 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*), it was not affected by drought simulation in our preliminary examination (*Matoša Kočar et al. 2022*), or the subsequent one (*Markulj Kulundžić et al. 2022*). Furthermore, TR_0/ABS values in the preliminary examination (*Matoša Kočar et al. 2022*) were very near the value considered optimal (0.83) for most of the plant species, according to *Björkman and Demmig-Adams* (*1995*), which 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, Tsimilli-Michael and Strasser 2008*). In the subsequent examination (*Markulj Kulundžić et al. 2022*), correlation analysis indicated TR_0/ABS was insensitive to RWC reduction and the increase of thiobarbituric acid reactive substances (TBARS) in leaves.

ChlF detecting mycorrhizal biofertiliser effect in alleviating drought stress

PI_{ABS} and PI_{total} were found to be significantly influenced by drought treatment in a preliminary plant pot experiment investigating the effect of mycorrhizal biofertilisers as a promising approach to alleviate drought stress. The experiment was conducted at the AIO in cooperation with the Chair for agricultural melioration of the Department for plant production and biotechnology from the Faculty of Agrobiotechnical Sciences Osijek. One soybean genotype was examined in five treatments with five replications. The soil in the control treatment was maintained at 80% AWC throughout the vegetation. Drought was initiated at the R1–R2 and R5–R6 growth stages, with and without mycorrhizal biofertiliser. ChlF was measured along with leaf temperature and chlorophyll content from the day the water was withheld until the water content in the soil reached 50% AWC at R1–R2 and R5–R6. According to average PI_{ABS} values (**Figure 1**), mycorrhizal biofertiliser helped mitigate the negative drought effects on the functioning of the photosynthetic apparatus at R1–R2. The differences between drought treatment with and without mycorrhizal biofertiliser at R5–R6 were noted only on the third day of withholding water. However, there was no significant variability between treatments in average PItotal at R1–R2 and no effect between drought treatments with and without mycorrhizal biofertiliser at R5–R6, either in average or by days.

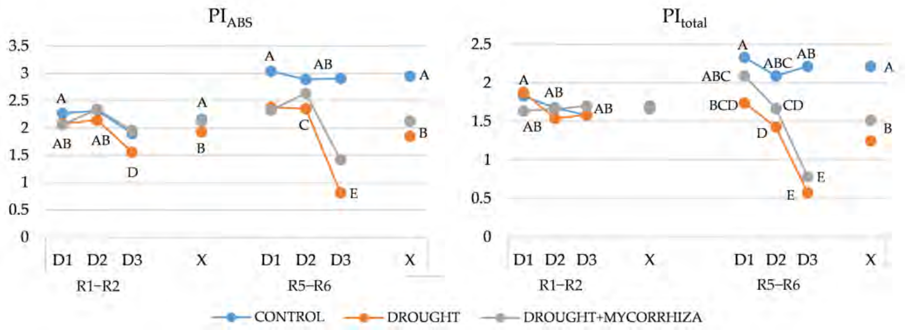

Figure 1. PIABS and PItotal measured on one soybean genotype in a plant pot experiment investigating mycorrhizal biofertiliser effect in alleviating drought stress during three days (D1–D3) at R1–R2 and R5–R6 growth stage and respective average values per growth stage (X). Data points per respective growth stage marked with the same capital letters are not significantly different (Fisher's LSD test, P<0.01) (source: Matoša Kočar M, Marković M, 2023, preliminary results – unpublished data).

ChlF detecting Cd stress

Besides using ChlF for determining the effects of drought stress, the same tool was used for detecting stress at the R3 growth stage caused by Cd contamination (*Josipović et al. 2014*). Increasing heavy metal rhizosphere pollution due to anthropogenic activities (*Masindi and Muedi 2018, Vareda et al. 2019*) necessitates research on its effects on plants. Cd accumulation in soybean plants is known to cause chlorosis and atrophy, difficulties in nutrient uptake, chloroplast structure damage, and reduced nodulation efficiency (*Pagani et al. 2012, Sun et al. 2012, Dias et al. 2013*). It can affect photosynthetic activity, antioxidant activity, plant water status, and redox imbalance (*Perfus-Barbeoch et al. 2002, Ortega-Villasante et al. 2005, Ekmekçi et al. 2008*). To determine genotype susceptibility to Cd stress, six soybean genotypes were grown in plant pots with four levels of cadmium contamination (0, 0.5, 1 and 5 mg Cd kg^{-1} soil). The variability of calculated PIABS values indicated it was sensitive enough to detect differences in physiological responses to Cd contamination (*Josipović et al. 2014*).

ChlF genotype screening in field conditions

Although greenhouse plant pot experiments are useful for determining ChlF parameters most indicative of abiotic stress or confirming growth stages in which soybean is especially susceptible to stress, they cannot substitute field trials in determining stress-susceptible or stress-tolerant genotypes. As the main economic consequence of stress susceptibility is yield loss, field trials are crucial for evaluating how physiologically determined stress reflects on grain yield. However, most of the ChlF studies were conducted in greenhouses, thus excluding most environmental variations present in field conditions (*Dong et al. 2019, Lotfi et al. 2019, Sakoda et al. 2022*). There are few reports on the use of ChlF for determining stress susceptibility in the field conditions (*Elsalahy and Reckling 2022, Nendel et al. 2023*), but only field trials can provide accurate and realistic assessments necessary for making informed decisions in genotype selection.

As fluctuating growing seasons are providing good research opportunities, we at the Agricultural Institute Osijek have recently established an ongoing field ChlF screening of our commercial cultivars and elite breeding material. To evaluate the differences in the photosynthetic efficiency between genotypes, ChlF is measured during flowering (R1–R2) and pod filling (R5–R6) in which soybean is the most susceptible to stress (R1–R2, R5–R6) according to previous studies (*Board and Kahlon 2011, Cui et al. 2019, Matoša Kočar et al. 2022*). Drought during these periods can reduce soybean yield by 30–80% (*Brown et al. 1985, Eck et al. 1987, Desclaux et al. 2000*). Furthermore, these growth stages coincide with periods when drought occurs more frequently in Croatia. ChlF data collected during the vegetation period is correlated with the data for yield and yield components.

ChlF detecting heat stress in field conditions

Along with our ongoing ChlF genotype screening in non-stress conditions, a heatwave occurring during the R5–R6 growth stage in 2022 provided an opportunity for evaluating the photosynthetic response to potentially stressful conditions of elevated temperatures and low air humidity by measuring ChlF in the morning and at midday. The weather conditions in the morning were considered a baseline for comparison, while the conditions at midday simulated potential stressful conditions (**Table 1**). The measurements were made at the AIO experimental field on two occasions, and weather conditions were measured by the Pinova Meteo agriculture weather station located near the site of the experiment. The preliminary results (**Figure 2**) indicate there was variability in genotype reaction to adverse conditions characterised by high temperatures and low relative air humidity. However, grain yield was in a very weak correlation with PI_{ABS} and PI_{total} measured in the morning (r = 0.16 and 0.05, respectively), and in a moderate and weak correlation with PI_{ABS} and PI_{total} measured at midday (r = 0.59 and 0.27, respectively).

Table 1. Average air temperatures and relative air humidity measured during the morning (8:00–9:00 hours) and at midday (12:00–13:00 hours) at the Pinova Meteo agriculture weather station.

	Day one		Day two	
	Morning	Midday	Morning	Midday
Average temperature (°C)	27.9	29.5	27.3	31.4
Relative air humidity (%)	49.7	39.97	38.1	21.85

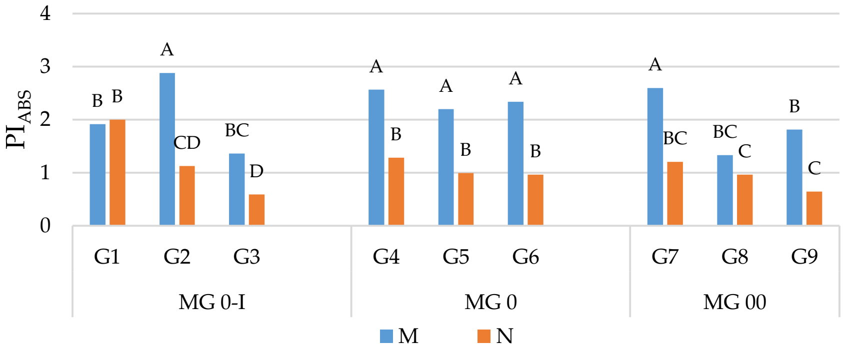

Figure 2. Average two-day PI_{ABS} values measured at R5–R6 growth stage in the morning (M) and at noon (N) on nine soybean genotypes (G1–G9) from three maturity groups (MG). Data points per respective MG marked with the same capital letters are not significantly different (Fisher's LSD test, P<0.05) (source: Matoša Kočar M, 2023, preliminary results – unpublished data).

ChlF detecting nitrogen-fixing bacteria effect

Grain yield was not correlated with PI_{ABS} or PI_{total} according to the preliminary two-year results of the field trial with four different nitrogen-fixing bacteria inoculants tested on three soybean cultivars at the AIO. As yield evaluation cannot be made based on the results of only one or two years, continuous trials are crucial for correct assumptions. The preliminary results of the above-mentioned ongoing field trial with bacterial inoculants indicate no significant differences between the average PI_{ABS} or PI_{total} values for individual treatments, neither at R1–R2 nor R5–R6 growth stage. However, there were significant differences between genotypes in average PI_{ABS} and PI_{total} measured at the R5–R6 (**Figure 3a**).

As can be seen from **Figure 3b**, the average PI_{ABS} and PI_{total} values per genotype in different treatments were very similar, except for T3. This leads to the conclusion that different inoculants did not impact photosynthetic efficiency.

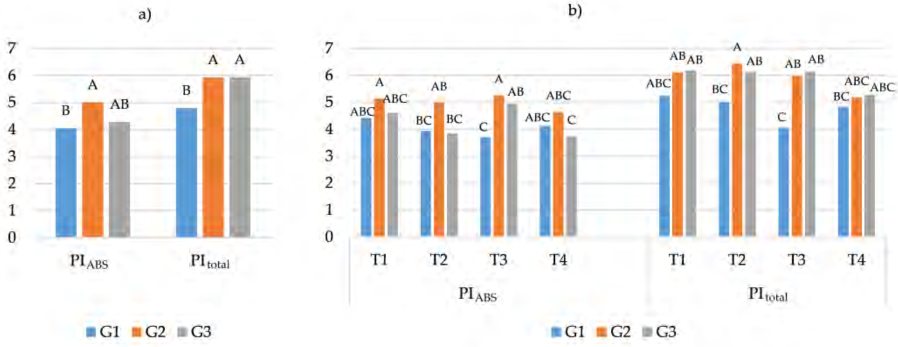

Figure 3. Average two-year PI_{ABS} and PI_{total} values measured at the R5–R6 growth stage on three soybean genotypes (G1–G3) and four nitrogen-fixing bacteria inoculants (T1–T4): a) average genotype values; b) average values per genotype and treatment. Data points per respective parameter marked with the same capital letters are not significantly different (Fisher's LSD test, $P<0.05$) (source: Matoša Kočar M, Duvnjak T, 2023, preliminary results – unpublished data).

Prospects for future ChlF use at the AIO soybean breeding programme

Overall, the main use of ChlF in soybean breeding at the AIO is for evaluating the adaptive capacity of genotypes to changing climate conditions. This should aid in creating stable and adaptable cultivars tolerant to abiotic stress. Creating more adaptable cultivars should increase the average crop productivity because stressful conditions are expected to occur in almost every growing season. According to *Buezo et al.* (*2019*), the high phenotypic plasticity among soybean should be exploited to compensate for the low genetic variability when selecting for superior productivity in constrained environments. However, ChlF parameters represent complex physiological processes, and their relationship with grain yield is, unfortunately, still not investigated enough, so their use as effective selection criteria for creating cultivars with superior productivity will necessitate further research. Nevertheless, ChlF is tentatively being utilized as a phenotyping tool for screening early generations in the AIO soybean breeding programme. The insights from our previous ChlF studies, together with relevant literature references, help with interpreting the data collected on the early-generation materials. ChlF data, combined with other observations in comparative phenotyping, is utilized for evaluating the photosynthetic efficiency in non-stress conditions and excluding the inferior genetic materials, thus facilitating early-generation selection. Naturally occurring drought conditions during the growing season enable us to evaluate the response of our early-generation materials to stress and provide us with additional information for decision-making. Nowadays, most of the knowledge on drought-stress tolerance is based on cumulative drought-stress research (low, frequent precipitation during the growing season; *Elsalahy et al. 2020, Gao et al. 2020, Saleem et al. 2022*), but genotype response to episodic drought (prolonged period of no precipitation), which is occurring more frequently in Central Europe (*Hari et al. 2020, Elsalahy and Reckling 2022*), needs to be investigated as well. Along with genotype response during episodic drought, the area of research that needs more attention is soybean potential for photosynthetic recovery after stressful conditions are terminated. According to *Elsalahy and Reckling* (*2022*), drought-tolerant soybean cultivars may partially be drought-resilient due to the recovery of photosynthetic traits. However, correlating the variability in the potential for growth compensation after stress with photosynthetic recovery evidenced by ChlF analysis necessitates more research conducted in field conditions. Furthermore, empirical and robust quantification of resilience to drought necessitates assessing the temporal dynamics of stress effect during plant growth, which is enabled by ChlF measurement (*Elsalahy and Reckling 2022*). Determining and confirming the ChlF traits that sustain soybean's high productivity under stressful conditions, i.e. selecting genotypes with superior functioning of the photosynthetic apparatus, enhanced photoprotective defenses, and higher overall productivity, would greatly increase the breeding efficiency in GMO-free conventional breeding programs, without increasing the cost of the inputs much beyond the cost of the device for ChlF measuring. Drought-tolerant soybean cultivars would enable resilient and adaptable crop production systems in Europe and thus reduce Europe's dependency on imported soybean.

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Insight into the Physiological Mechanism of Sunflower Plants from the Aspect of Chlorophyll *a* Fluorescence

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Scientists at the Agricultural Institute Osijek (AIO) started growing sunflower plants, as well as conducting breeding, and studying the genetics of sunflower plants, back in the 1970s. The twentieth century was recorded as a period of economic growth but also as a period of intensive study of plant mechanisms, which was followed by the development of new methodologies in plant research (*Nagy and Garab 2021*). The implications of the latest scientific knowledge on the regulatory mechanisms of plants were among the most critical goals in photosynthesis research. Today, measurements of photosynthetic parameters have become widely accepted basis of genetic resources research. They were included in the breeding of modern varieties and the technological process improvement in crop cultivation worldwide (*Brestic et al. 2021*). It is known that the photosynthetic efficiency of plant species depends not only on their genetic potential to absorb light energy and utilize it for the production of carbohydrates but is also affected by numerous environmental factors (*Andrianasolo et al. 2016*) that are global challenges to growing crops. Previous research on sunflower plants focused on the influence of drought (*Ghobadi et al. 2013, Umar et al. 2019, Çiçek et al. 2019, Arslan et al. 2020*), high temperatures (*Killi et al. 2020*), light (*Cinq-Mars and Samson 2021*), salinity (*Liu and Shi 2010, Umar et al. 2019, Ahsan et al. 2023*), mineral nutrition (*Ohnishi et al. 2021, Ma et al. 2022*), CO_2 concentration (*Takagi et al. 2016*), a combination of drought and heat stress (*Killi et al. 2020*), etc. on JIP test parameters from chlorophyll *a* fluorescence transient OJIP. The sunflower breeding program at the Department of Industrial Plants Breeding and Genetics at the AIO followed the research trends, and accordingly, with the advent of chlorophyll *a* fluorescence (ChlF) and some physiological parameters, the scientists decided to test the aforementioned analysis on sunflower plants grown in the Osijek-Baranja County location. According to the literature, common to all the conducted research, both by other authors and in our research, is the testing of sunflower genotypes, i.e. lines and hybrids, to find out their sensitivity to the tested conditions. Also, the purpose of such research is to determine whether ChlF analyses can be used in breeding programs as a method of material selection beneficial to shorten the process of creating a genotype with traits of interest.

The reaction of plants to stress is manifested through changes in biochemical and physiological cascades that range from photosynthesis to metabolic processes. Plants show different responses to drought stress, which differ depending on the level observed, whole plant, cellular, and molecular levels (*Hussain et al. 2018*). Accordingly, *Arslan et al. (2020)* examined the impact of one of today's biggest problems in crop production, drought. Under controlled conditions, they conducted an experiment on sunflower lines, inducing a drought that lasted for 10 days. After exposure to drought, the sunflower lines were watered for 5 days. The goal was to monitor the functionality and structure of the integrity of the photosynthetic apparatus using ChlF measurements. The authors found that the drought had a significant effect on all ChlF parameters as quantum yields (flux ratio) and specific energy fluxes, as well as that drought adversely affected the photosynthetic activity of all lines. The maximum quantum yield of PSII photochemistry (ϕP_0; F_v/F_m; TR_0/ABS), quantum yield for electron transport at t = 0 (ϕE_0; ET_0/ABS), the quantum yield of electron transport from Q_A^- to the PSI end electron acceptors (ϕR_0; RE_0/ABS) and probability with which an electron from the intersystem electron carriers move to reduce end electron acceptors at the PSI acceptor side (δR_0; RE_0/ET_0), performance index (PI_{ABS}) and the photosynthetic efficiency index of energy required from exciton to reduction of ultimate electron acceptor on photosystem I (PI_{total}) decreased under water deficit, indicating the obstacles of transferring electrons to the electron transport chain. Conversely, absorption flux per reaction centre (ABS/RC) and dissipation energy flux per RC (DI_0/RC) increased in drought. Such results in sunflower cultivars were also obtained by *Umar et al. (2019),* who suggested that fractions of RC (ABS/RC and DI_0/RC) became dissipative centres to prevent photooxidative damage to the photosynthetic apparatus. However, after re-watering, the recovery of photosynthetic activity showed that the drought treatment did not cause irreversible damage to the photosynthetic apparatus. Furthermore, examining the effects of moderate and severe drought on sunflower cultivars, *Ghobadi et al. (2013)* found significant differences among cultivars in terms of F_v/F_m and PI of the most commonly used ChlF parameters in testing the effects of stress, whose values significantly decreased in response to drought. In the flowering stage, drought led to a decrease in Fv/Fm and PI, while in the mid grain filling stage, the values of Fv/Fm and PI were lower, but the effect of drought treatment on Fv/Fm and PI was greater than in the flowering stage. *Hussain et al. (2018)* stated that under drought stress, stomatal closes and CO_2 fixation decreases, which results in reduced photosynthesis in sunflowers. Limited diffusion of CO_2 in sunflower leaves mediated by stomatal closure is more dominant compared to CO_2 assimilation. Photosynthetic damage caused by drought can be compensated by increased CO_2 levels.

Motivated by the problems in crop production, scientists at the AIO also researched the effect of water deficit on the physiology of sunflower plants. We conducted research in a greenhouse on sunflower hybrids that were maintained at two water levels, 60 and 80% of field water capacity (FWC), at four different stages of sunflower plant development. *Markulj Kulundžić et al. (2016a)* studied PI_{ABS} and leaf temperature on 13 sunflower hybrids at the V6 stage of development. Differences between the treatments (60 and 80% FWC) were not determined, probably because, at that stage of development, the specified soil saturation with water does not affect PI_{ABS}. In contrast, significant PI_{ABS} differences were found between hybrids within treatments as a result of their genetic divergence. In the treatment with lower water content, all hybrids resulted in an increase in leaf temperature due to lack of water. In the research in the butonization stage (R2), *Markulj et al. (2014)* did not determine a significant difference for F_v/F_m considering water stress conditions, which *Killi et al. (2020)* established. The opposite of that, PI_{ABS}, as a parameter more sensitive to environmental conditions, showed a difference between treatments, which was also confirmed by *Ghobadi et al. (2013).* As a follow-up to these studies, tests were carried out on the influence of water content in the soil in the flowering (R3/R4) and grain filling (R6) stages of development when an increase in F_v/F_m and PI_{ABS} values was found in most genotypes, which confirmed the slight influence of water deficit on the photosynthetic apparatus in those stages (*Markulj Kulundžić et al. 2016b, Markulj Kulundžić et al. 2022a*). Interestingly, the values of F_v/F_m and PI_{ABS} in drier conditions at the initial stages of sunflower growth and development (V6 and R2 stage) decreased, and at R3/R4 and R6 stages increased, which indicated that the photosynthetic processes in sunflower leaves depend on the time of onset of drought. Those parameters' behaviour was in contrast to the results of *Ghobadi et al. (2013).* Also, observing the results by the stages of plant development, similar F_v/F_m values were observed, but an increase was recorded in PI_{ABS} values with the aging of sunflower plants, which was also confirmed in wheat (*Viljevac Vuletić et al. 2019*). In the aforementioned experiments, the focus was on the two most sensitive ChlF parameters to get a quick insight into the physiological response of plants to drier soil conditions.

Furthermore, studying the literature, we saw that the interaction of light and temperature has rarely been a subject of research. Guided by that, we conducted a study for a doctoral dissertation entitled “Biochemical regulatory mechanisms of photosystem II efficiency in sunflower leaves affected by the environmental stress” (*Markulj Kulundžić 2019*) that examined the effect of elevated temperatures and excess light in combination. Seeing that most scientists conduct research in controlled conditions, we decided to examine the effect of elevated temperatures and excess light in field conditions, which gave insight into cultivation in real environmental conditions, making the data useful to agronomists. As in the previous papers, we approached the research on sunflower hybrids of the AIO in more detail, and we included properties for the detection of oxidative stress and antioxidant enzymes. The study consisted of two parts. The first part included testing relative water content, lipid peroxidation levels, guaiacol peroxidase enzyme activity, photosynthetic pigment concentrations, photosynthetic efficiency, and grain yield on eight sunflower hybrids in the morning and afternoon conditions. The second part of the study included a further study on the highlighted two hybrids, based on results of the first part of the study, in the form of insight into the polyphasic growth of chlorophyll *a* fluorescence and the relative accumulation of photosystem II light-harvesting complex proteins Lhcb2, D1 of photosystem II, cytochrome *f* and the large subunit of Rubisco, also in the morning and afternoon conditions. Measurements in the morning conditions were implied by the control conditions, while measurements in the afternoon conditions were considered as an influence of elevated temperatures and excess light. All investigated parameters in the tested hybrids in the flowering stage showed significant differences with large fluctuation values, but there was a visible lack of clearly general regularity in conditions without stress and during heat and light stress. The elevated temperature and excess light in the early afternoon led to changes in chlorophyll fluorescence, which resulted in damage to the photosynthetic apparatus on all eight tested hybrids, including the two sunflower hybrids selected for further research. F_v/F_m and PI_{ABS} values of both selected hybrids decreased during heat and light stress. *Mlinarić et al. (2016), Mlinarić et al. (2017), Chen et al. (2017),* and *Mihaljević et al.* (*2020*) confirmed those results. A more pronounced decrease was in hybrid 7 (F_v/F_m below 0.75), which indicated damage to photosystem II in terms of disruption of its primary photochemistry. A significant increase in ABS/RC in both hybrids under stress conditions and a decrease in the ratio of chlorophyll *a* and *b* concentrations indicate an increase in the size of photosystem II antennae. This may be due to the high proton gradient across the thylakoid membrane under stress conditions, causing the active reaction centre to become a dissipative centre and convert violaxanthin to zeaxanthin, thereby preventing oxidative damage under extreme conditions (*Umar et al. 2019*). The increase in the value of this parameter was also significantly more pronounced in hybrid 7 due to the higher potential of the relative accumulation of the light-harvesting protein (Lhcb2) compared to hybrid 4. At the same time, hybrid 7 showed a more pronounced increase in the trapping per active reaction centre (TR_0/RC), both in non-stressed and stressed conditions, than hybrid 4, which indicated a higher ability of its reaction centres to utilize absorbed light energy. An increase in both mentioned parameters led to an increased flow of electrons into the electron transport chain, resulting in its oversaturation, which is reflected in a drop in the value of the electron transport parameter away from the primary acceptor Q_A ($ET_0/(TR_0-ET_0)$), and ultimately in lowering the value of PI_{ABS}.

Following on from the previous research, *Markulj Kulundžić et al. (2021)* published results that described ChlF transients in the light phase of photosynthesis during the combined effect of increased temperatures and high irradiation in rainfed and irrigated conditions in sunflower plants. The results showed the influence of irrigation only on PI_{ABS} in 2013 and TR_0/ABS in 2014. According to TR_0/ABS, half of the tested sunflower hybrids had disturbed photosynthetic processes in the combination of increased temperatures and high irradiation because TR_0/ABS was reduced in the afternoon conditions. These values did not go below 0.75, which emphasised the impairment of the photochemical efficiency of photosystem II (*Sharma et al. 2015*). Also, PI_{ABS} values, as well as electron transport per active reaction centre (ET_0/RC), decreased due to increased temperatures and irradiation light, in contrast to increased values of ABS/RC, TR_0/RC and DI_0/RC, which impaired the functioning of the photosynthetic apparatus. The same pattern of the mentioned parameters under heat and light stress was also established by *Mihaljević et al. (2020)* and *Mlinarić et al. (2016).* A multivariate principle component analysis (PCA) was used to display the relationship between the results (**Figure 1**), which has been popular in recent years to assess the relationship between ChlF parameters and other properties (*Galić et al. 2020, Viljevac Vuletić and Španić 2020, Zhu et al. 2021, Markulj Kulundžić et al. 2022, Matoša Kočar et al. 2022*).

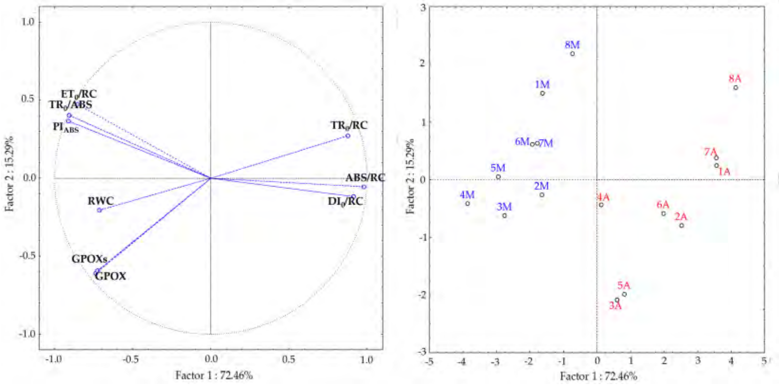

Figure 1. Biplots are constructed based on the results of the principal component analysis for thirteen parameters and eight sunflower hybrids (source: *Markulj Kulundžić et al. 2021).*

The study by *Markulj Kulundžić et al. (2022c)* also shows the photosynthetic response of two sunflower hybrids to elevated temperatures and excess light in the flowering stage by ChlF measurements in the morning and afternoon field conditions to determine the content of photosynthetic pigment and the relative accumulation of photosynthetic proteins. Fluorescence transient curves were used to display the results, all of which are present today to describe the OJIP steps (**Figure 2**).

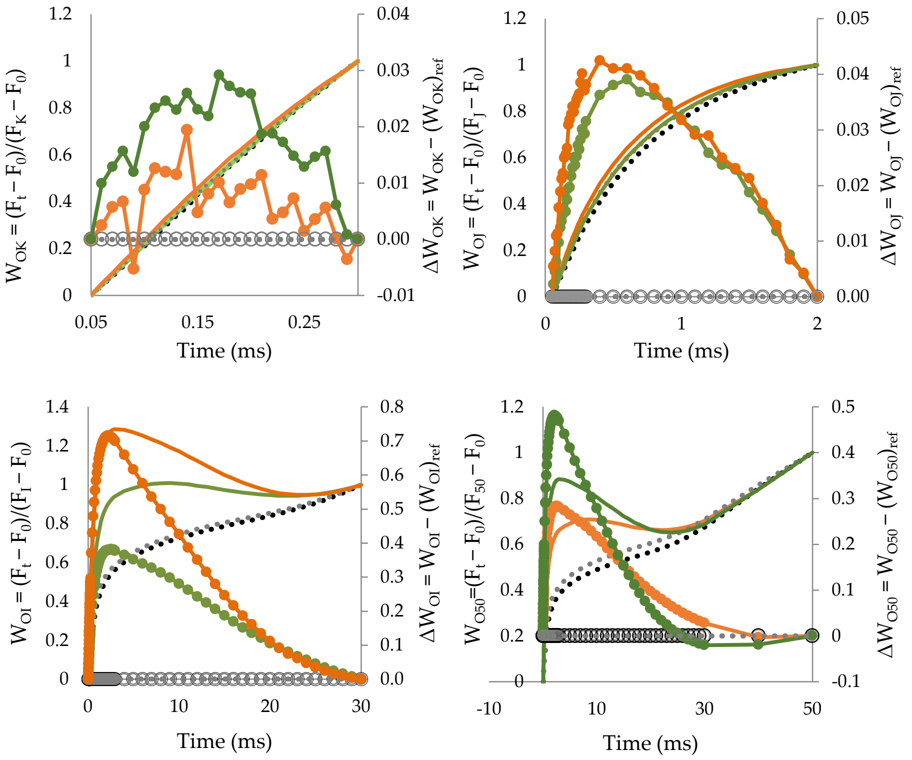

Figure 2. Chlorophyll *a* fluorescence transient curves in sunflower hybrids 4 and 7 in the morning and afternoon conditions (source: *Markulj Kulundžić et al. 2022c)*

Under the synergistic effect of elevated temperatures and excess light, positive L, K, J, and I steps appeared, indicating a weaker energy connection and stability of PSII units, the weakened function of the PSII antenna during electron flow, and a decrease in the amount of plastoquinone between PSII and PSI (*Yusuf et al. 2010, Papageorgiou and Govindjee 2014, Paunov et al. 2018*). As expected, most of the tested parameters changed during elevated temperatures and excess light, but only F_0 (minimal fluorescence), the energy flow that reduces electron end acceptors on the acceptor side of the photosystem I (RE_0/RC), and D1 showed significant, high and positive correlations with environmental conditions, which indicates their usefulness in the study of sunflower response to heat stress. Relative variable fluorescence at 3 ms (V_J step), TR_0/ABS, flux ratio trapping per dissipation (TR_0/DI_0), $ET_0/(TR_0-ET_0)$, RE_0/ABS, $RE_0/ET0$, RE_0/RC, Lhcb2 and D1 can also be used as indicators of physiological changes under conditions of elevated temperatures and excess light, despite their indirect connection with environmental conditions.

One of the interests of the researchers was the connection of ChlF parameters with yield components, such as – F_0, F_m (maximal fluorescence), V_J, V_I (relative variable fluorescence at 30 ms), F_v (variable fluorescence), F_v/F_m, ABS/RC, DI_0/RC, TR_0/RC, ET_0/RC, RE_0/RC, RE_0/ET_0, RC/ABS (quantum yield for reduction of end electron acceptors at the PSI acceptor side), TR_0/DI_0, $ET_0/(TR_0-ET_0)$ and PI_{ABS} with seed weight per head, plant height, head diameter, number of head seeds and 1000-grain weight (*Markulj Kulundžić et al. 2023*). Given that the existing methods for improving yields have been exhausted, today's widespread study of photosynthesis, which plays a very important role in determining yields, should be linked to agronomic traits to support future requirements for increasing the crop yields (*Monteoliva et al. 2021*). Growth rate and productivity are crucial factors in crop yields whose variations can cause variations in photosynthetic efficiency (*Flood et al. 2011*). Namely, in our study, the significance of ChlF parameters (except for PI_{total}), chlorophyll content (SPAD), leaf temperature, and agronomic traits, was confirmed between the hybrids. Indicators of photosynthetic efficiency showed a significant correlation of plant height with V_I, RE_0/ET_0, and PI_{total}. Also, the number of seeds per head showed a positive and very significant correlation with V_I and a very high significant correlation of negative direction with RE_0/RC. Successes in positively identifying photosynthetic parameters associated with crop biomass accumulation have been rare (*Qu et al. 2017*). *Driever et al. (2014)* did not establish a correlation between grain yield and photosynthetic capacity despite signification variations in wheat's photosynthetic capacity, biomass, and yield.

During the last few years, we have also been dealing with the effects of fertilization and different types of soil on the development and photosynthesis of sunflowers, as well as the examination of photosynthesis in other stages of growth and development of sunflowers with extended biochemical analyses, but we have not published these data yet.

A common thing in the mentioned papers was the examination of sunflower genotypes (line/hybrid) using ChlF parameters to determine their sensitivity. All authors determined sensitive and tolerant genotypes to the tested stress conditions. Less damage to the photosynthetic structure resistant to the stressor was found in more tolerant genotypes. Therefore, these genotypes maintained their photosynthetic performance because they had smaller changes in energy flows, which approximately control their levels under the effect of stress, which is the opposite for sensitive genotypes. It is believed that the technique of chlorophyll *a* fluorescence measurement can be utilized in breeding programs along with molecular techniques for a large number of hybrids in field conditions (*Çiçek et al. 2019*).

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Unveiling the Potential of Chlorophyll *a* Fluorescence as a High-Throughput Phenotyping Method in Maize Breeding and Research

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Maize (*Zea mays* L.) is one of the most important crops in the world and is widely used for food, feed, and industrial purposes. In order to improve important traits such as yield, stress tolerance, and quality of maize, it is important to understand the factors that affect its growth and development. Crop response to different stresses affected by weather anomalies is highly complex and involves changes at the genetic and physiological levels that facilitate avoiding and/or coping with stress (*Galić et al. 2019*). In agronomy, the stress-avoiding strategy is commonly applied and stress can be circumvented by agricultural practice (*Jung and Müller 2009, Teixeira et al. 2013*). On the other hand, modern plant breeding aims to conduct studies on trait physiology, phenotyping, and genotyping on how to cope with stress (*Araus et al. 2012, Fahad et al. 2017*). Coping with climate change, from a crop breeding perspective, demands phenotypes that are heritable, easily measured, and show specific responses to different stressful conditions often found in growing environments (*Franić et al. 2020b*). An understanding of the physiological reactions of plants to stress is essential for the development of crops with improved stress tolerance.

Crop phenotyping is a crucial step in maize breeding as it allows breeders to assess the performance of their breeding material and make informed decisions about which lines to advance to the next generation or which hybrids to choose for commercial production. Phenotyping tools and applied methods are used to evaluate and measure complex traits related to growth, yield, quality, and adaptation to different environmental stresses (*Kondić-Špika et al. 2022*). High-throughput methods sensitive to various stresses are needed (*Franić et al.*

2020b). In recent years, chlorophyll a fluorescence analysis has been found to be a useful phenotyping tool, as it provides a non-invasive and fast way to measure physiological status of plants and generates a considerable amount of data belonging to high-thro-phenotyping methods (*Galić et al. 2019*). By measuring the chlorophyll *a* fluorescence, researchers can gain insights into the functioning of the photosynthetic machinery, which is important for understanding the effects of environmental factors on plant growth and development. Every environmental change forces the photosynthetic apparatus to adjust its physiological state and parameters of fast polyphasic fluorescence induction transient change accordingly (*Strasser et al. 2004*).

In the last few decades, analysis of chlorophyll *a* fluorescence has been studied for its potential use as a tool for improving crop productivity and stress tolerance. It has been a widely used method to evaluate the health or integrity of the internal apparatus during the photosynthetic process within a leaf (*Clark et al. 2000*). Photosynthesis is a crucial process in plants, as it is responsible for converting light energy into chemical energy. Studies have shown that stress can have a significant impact on photosynthesis in maize, leading to a decrease in its efficiency (*Schreiber et al. 1994, Brestic and Zivcak 2013*). For example, water stress can result in a decrease in stomatal conductance, which reduces the amount of carbon dioxide that can be taken up by the plant, leading to a decrease in photosynthesis (*Ghannoum 2009*). High temperatures can also cause a reduction in the efficiency of photosynthesis by reducing the activity of photosynthetic enzymes (*Hu et al. 2020*). However, the relationship between photosynthesis and stress in maize is not straightforward, as the effects of stress can vary depending on the type and severity of the stress factor, as well as the genotype and developmental stage of the plant.

A method of fast chlorophyll *a* fluorescence transient measuring (OJIP) developed by *Strasser and Govindjee (1992a, 1992b)* can be translated via JIP test into several phenomenological and biophysical parameters (*Strasser et al. 1995, Strasser et al. 2000, Strasser et al. 2004*) that quantify photosystem II functioning and can reflect the activity of the whole photosynthetic machinery (*Strasser et al. 2004*). One of the most often used parameters are the maximum quantum yield of photosystem II (F_v/F_m) and performance indexes (PI_{ABS} and PI_{total}) taking into the account all of the main photochemical processes. PI_{ABS} appears to be a very suitable and sensitive parameter to investigate plant overall photosynthetic performance under different abiotic and biotic stresses under controlled and field conditions (*Reddy and Strasser 2000, Živčák et al. 2008, Šimić et al. 2014*). This is particularly important for crop improvement because stress studies conducted under controlled conditions inadequately reflect the natural environmental conditions (*Galić et al. 2019*).

Numerous research studies on fluorescence have been conducted in maize to understand the underlying mechanisms and benefits of this process. At the

Agricultural Institute Osijek, analysis of chlorophyll *a* fluorescence in maize has been used since 2009 to study the effects of various environmental factors on maize growth and development, including drought, temperature, salinity, and heavy metal toxicity.

In 2009 *Lepeduš et al. (2012)* conducted preliminary research to determine differences in photosynthetic efficiency under water-limited conditions among flint and dent maize inbred lines belonging to various heterotic groups. Chlorophyll *a* fluorescence measurements were taken in the field during silking by Handy PEA (Hansatech, King's Lynn, UK), and the data obtained were used to calculate two biophysical parameters that describe the photochemistry of photosystem II: F_v/F_m and performance index on absorption basis (PI_{ABS}). There were significant differences among inbred lines for both investigated chlorophyll *a* fluorescence parameters, but differences in photosynthetic efficiency were higher within dent inbred lines than between dents and flints. Inbred lines B73 and Mo17 differed remarkably for F_v/F_m and PI_{ABS} parameters under the very dry scenario, thereby providing contrasting parents for the mapping population, which can be an excellent resource for further photosynthesis studies.

In 2010 and 2011, *Šimić et al. (2014)* conducted a quantitative trait loci (QTL) analysis for nine JIP test parameters in maize during flowering in four field environments, differing in weather conditions, to identify putative candidate genes which might help to explain the genetic relationship between photosynthesis and different field scenarios in maize plants. The intermated B73_Mo17 (IBM) maize population of 205 recombinant inbred lines was used for this research. In 2010 the two parents differed considerably in the chlorophyll fluorescence parameters when grown under very dry but not under dry conditions, indicating that chlorophyll fluorescence can be an efficient and sensitive selection tool for different drought scenarios. Further investigations done in 2011 revealed that there is also a considerable influence of irradiation on photosynthetic performances in the two parents grown under well-watered conditions. The JIP test parameters showed to be under different genetic controls in different environments. QTL analysis was capable of detecting 10 significant loci on chromosomes 1, 5, 7, and 8, coinciding with the genes possibly associated with chlorophyll *a* fluorescence parameters in different field environments. However, many of the QTLs were not stable across environments indicating the "adaptive" nature of QTLs. One pleiotropic locus was detected on chromosome 7, coinciding with the gene gst23, which may be associated with efficient photosynthesis under different field scenarios.

Since photosynthesis is the basis of yield formation, it is important to know at which plant densities it is significantly affected. In order to estimate the effect of plant density on agronomic traits and photosynthetic efficiency, *Franić et al. (2015)* conducted further research on the IBM population in the field during 2013. Mean values of grain yield per plot and photosynthetic performance index were higher in normal density, whereas grain yield per hectare was higher in high density. A lower performance index in higher density indicated that plants were stressed due to increased mutual plant shading, which caused depression in photosynthesis and greater competition for water and nutrients (*Marchiori et al. 2014*). Higher plant density affected grain yield per plant, but lower yields per plant in high density were compensated by a larger number of plants per hectare, resulting in higher yields on a yield per hectare basis. These results are in accordance with *Gonzalo et al. (2010)* research on the response of 186 B73xMo17 recombinant inbred lines to low and high density.

Chlorophyll *a* fluorescence measurement proved to be a suitable method for the detection and evaluation of heavy metal stresses (*Żurek et al. 2014*), and it is sensitive to cadmium (Cd) stress (*Larsson et al. 1998, Di Cagno et al. 1999, Burzyński and Żurek 2007*). It is generally assumed that toxic trace metals enter plant cells through transporters of essential metals, and trace metal uptake is in competition with the uptake of essential metals, such as K, Ca, Mg, Fe, and Mn (*Franić and Galić 2019*). However, mechanisms of Cd uptake and translocation have not yet been properly elucidated. Cadmium is a non-essential metal that causes oxidative stress in plants by altering the activities of antioxidant defense mechanisms resulting in hydrogen peroxide accumulation (*Sandalio et al. 2001, Schützendübel and Polle 2002, Cho and Seo 2005*), which leads to an increase of protein (*Romero-Puertas et al. 2002*) and lipid peroxidation (*Chaoui et al. 1997).* Since plants possess no Cd-exclusion mechanism (*Gallego et al. 2012*), it is important to evaluate the effects of excessive cadmium content in soil on plants.

A large number of studies have been performed on the effect of Cd on photosynthetic machinery, but relatively few studies have focused on the influence of Cd on the photosynthetic apparatus in plants grown from seeds on soil polluted with Cd (*Baryla et al. 2001*). *Franić et al. (2018)* conducted research on young plants of maize inbred lines B84 and Os6-2 and their respective hybrid by means of chlorophyll *a* fluorescence and the activity of antioxidant enzymes to investigate different responses to an elevated Cd content in the soil. Plantlets were grown for ten days after planting, and chlorophyll *a* fluorescence measurements were performed. Probably due to a dilution effect, there was no or very weak Cd stress detectable through antioxidant enzyme activities or hydrogen peroxide content in the hybrid, but the stress was detected through chlorophyll *a* fluorescence measurements. In this study, chlorophyll *a* fluorescence was shown to be a more sensitive method for Cd-stress detection than the biochemical parameters examined, and the results showed these methods as complementary to each other. Maize inbreds have been shown to differ in the uptake of heavy metals (*Florijn and van Beusichem 1993, Brkić et al. 2003*), and temperate inbred lines B84 and Os6-2 have been designated as different according to their respective ionomic profiles (*Sorić et al. 2011, Šimić et al. 2012*) and leaf Cd accumulation (*Sorić et al.*

2009). Inbred line B84 showed to be a low, and Os6-2 a high Cd accumulator, their respective hybrid being intermediate (*Franić et al. 2013*).

While the impact of the individual stress factors on maize's photosynthetic efficiency has been well documented, there is a lack of information dealing with the multiple stress tolerance (*Malenica et al. 2021*). A combination of excess Cd and drought stress was tested in lines Os6-2 and B84, and their hybrid (*Mlinarić et al. 2018*). A decreased PI_{total} was reported in Os6-2 and the hybrid. The destabilization of the oxygen-evolving center of photosystem II and lower photosystem stability was also observed. Photoinhibition due to the stress combination also occurred in line B84, despite an unchanged PI_{total}. The results suggested that all the investigated maize genotypes have developed different strategies to cope with a combination of excess cadmium and drought.

In order to examine the variation of Cd uptake in maize leaves, detect the effects of Cd uptake on the photosystem of selected genotypes through chlorophyll fluorescence, and identify if there is possible tolerance or sensitivity of the selected genotypes to Cd, *Franić et al. (2020a)* conducted two-year pot vegetation experiments with four maize genotypes and four different soil Cd levels. Chlorophyll *a* fluorescence was measured in the first half of July (2012 and 2013) during flowering (tasseling) when maize plants are particularly susceptible to stress, using Handy PEA. Chlorophyll fluorescence, photosynthetic pigments, and inductively coupled plasma (ICP) analysis for ear-leaf Cd and zinc (Zn) and soil Cd were carried out. Increased levels of Cd in soil caused genotype-dependent changes in concentration and photosynthetic machinery detectable by changes in chlorophyll *a* fluorescence transients, JIP test parameter values, dry mass, and photosynthetic pigments. Selected genotypes were separated into two distinct groups by ICP analysis: high accumulating (Os6-2, Mo17) and low accumulating (B73, B84). Changes in chlorophyll fluorescence caused by elevated Cd levels in soil were mostly visible as an increase in dissipation energy (ϕD_0, DI_0/RC), decreased density of reaction centers (RC/ABS), and decreased contributions of light reactions and electron transport for primary photochemistry (TR_0/DI_0 and ($ET_0(TR_0–ET_0)$)). Consequently, decreases in performance indexes (PI_{ABS}, PI_{total}) were observed. Decreases in chlorophyll fluorescence parameters suggest reduced functionality of reaction centers and problems in the reoxidation of primary quinone acceptor (Q_A). One possible strategy to utilize these results in the maize breeding program could be to identify tolerant cultivars to Cd toxicity bred for the Cd-contaminated areas. The other would be to identify cultivars capable of high uptake of toxic trace metals without exhibiting toxicity for remediation of contaminated areas. However, studies with broader germplasm and high-throughput techniques are needed.

Various stresses have different effects on plants. Generally, heat stress causes unique physiological conditions: an increase in respiration, stomatal conductance, and leaf temperature, reduced activity of antioxidant enzymes, and an increase in ROS production (*Gong et al. 1997, Rizhsky et al. 2002, Rizhsky et al. 2004*). Cold stress inhibits metabolic reactions and induces osmotic, oxidative, and other stresses along with changes in photosynthetic activity (*Strauss et al. 2006, Chinnusamy et al. 2007).* The majority of abiotic stress experiments are conducted in controlled conditions and do not reflect the actual conditions that plants are subjected to in the field. The study by *Franić et al.* (*2020b)* aimed to investigate the effects of extreme temperatures and low light conditions on photosynthesis in young plants of five maize hybrids by measuring chlorophyll *a* fluorescence. Furthermore, the authors examined whether maize hybrids from different maturity groups show distinctive reactions when subjected to different stresses and investigated the informativeness of different fluorescence parameters in different stress conditions. Five maize hybrids were used in this study representing the maturity groups from FAO 300 to FAO 600. Young plants were subjected to cold, low light, heat, and severe heat treatments and field conditions along with control and chlorophyll *a* fluorescence was measured on the middle section of the first fully developed leaf using the fluorimeter Handy PEA. ChlF transients were shown to respond to different treatments by changing the appearance of the OJIP curve, mostly at steps J and I, while the appearance of the K step was confirmed as a good indicator of temperature stress. JIP test data were used to perform principal component (PC) analysis, which revealed grouping of parameters with PCs according to their informativeness. Efficiency/probability for electron transport (ψE_0) was shown to be most defined by the field treatment, probably due to higher light intensity compared to control. Variables regarding electron transport and dissipation showed specific reactions to heat and severe heat treatments. Cold and low light conditions caused specific changes in relative variable fluorescence at J step (V_J) and I step (V_I).

Soil salinity is a very common abiotic factor in crop production that negatively affects plant growth in the form of hyperosmotic and hyperionic stresses (*Gupta and Huang 2014, Kan et al. 2017*). Salt stress can reduce growth in an early phase of plant development, which significantly reduces the yield (*Zörb et al. 2018*). High Na^+ concentration inhibits the uptake of K^+ ions resulting in reduced productivity or even death (*James et al. 2011*). *Galić et al. (2020)* tested the responses of five 10 days old maize hybrids to salinity stress by measuring chlorophyll *a* fluorescence parameters, fresh (FM), and dry mass (DM). Chlorophyll *a* fluorescence data were incorporated into a penalized regression model to predict biomass traits. The values of FM and DM significantly decreased under salt stress. Strong responses in chlorophyll *a* fluorescence parameters, giving information on the heterogeneity of electron transport, photosystem II reaction centers, and overall photosynthetic performance, showed significant effects of the NaCl stress. Stable values of maximum quantum yield for primary photochemistry (ϕP_0) under salt stress indicate that NaCl treatment did not irreversibly damage the functioning of photosystem II. In penalized regression models, 118 transients showed greater, although comparable, predictive abilities as 18 selected JIP test parameters. Since chlorophyll *a* fluorescence kinetics is a highly informative tool used for studying the effects of different environmental stresses, including salt stress on photosynthesis, it might serve as a new, adequate, high-throughput phenotyping tool in crop growth models and genotype-to-phenotype models.

Although chlorophyll *a* fluorescence analysis has proven to be a useful tool for testing plant stress responses both in young plants under controlled conditions and as an indicator of stress during the flowering of maize in the field conditions, the relations between chlorophyll fluorescence parameters and grain yield in the field environments have not been sufficiently examined. *Kalaji et al. (2017)* pointed out the importance of obtaining chlorophyll *a* fluorescence-related traits showing a high correlation with yield or plant performance in addition to chlorophyll *a* fluorescence-related traits, specific for tolerance/resistance to the stress of interest. *Galić et al. (2019)* assumed that the use of certain chlorophyll *a* fluorescence parameters of the JIP test as secondary traits for selection under moderate heat stress would be an efficient breeding strategy if chlorophyll *a* fluorescence parameters would be genetically variable, genetically correlated with grain yield in the target environment, and not associated with any yield loss under non-stressed conditions. The testcrosses of 221 intermated recombinant inbred lines (IRILs) of the IBMSyn4 population were evaluated in six environments at two geographically distinctive locations (Croatia and Turkey) in 3 years. The two locations in Croatia and Turkey were categorized as mild heat and moderate heat scenarios, respectively. The most discernible differences among the scenarios were obtained for the efficiency of electron transport beyond quinone A (Q_A) ($ET_0/(TR_0-ET_0)$), PI_{ABS}, and grain yield. Under the moderate heat scenario, there were tight positive genetic correlations between $ET_0/(TR_0-ET_0)$, and grain yield, as well as between PI_{ABS} and grain yield. Associations between the traits were noticeably weaker under the mild heat scenario. Analysis of QTL revealed several common QTLs for photosynthetic and yield performance under the moderate heat scenario corroborating pleiotropy. Although the indirect selection with chlorophyll *a* fluorescence parameters is less efficient than direct selection, the authors considered that $ET_0/(TR_0-ET_0)$ and PI_{ABS} could be efficient secondary breeding traits for selection under moderate heat stress since they seem to be genetically correlated with grain yield in the stressed environments, and not associated with yield performance under non-stressed conditions predicting grain yield during flowering. Indirect selection through PI_{ABS} was also shown to be more efficient than genomic selection in a moderate heat scenario. These results demonstrated that chlorophyll *a* fluorescence via JIP test is an appropriate method for realizing real-time, non-destructive monitoring of maize performance during flowering in the field environments. It seems that chlorophyll *a* fluorescence parameters may be used for predicting grain yield when heat stress occurs during vegetative and reproductive growth stages, as well as secondary traits used for selection under moderate heat stress. However, in future quantitative genetic studies on more severe heat stress scenarios, it would be worthwhile to examine changes in genetic correlations between chlorophyll *a* fluorescence parameters and grain yield.

In many studies, JIP test has been proven useful in monitoring the plant status in relation to stress. However, this approach suffers from several limitations. The need for 30 minutes of dark adaptation of samples represents a limiting factor for the throughput of measurements. Furthermore, only a one-second measurement is used to assess the status of whole plant, and actinic light applied to induce the fluorescence does not resemble the sunlight plant is subjected to in the field conditions (*Vlaović et al. 2020*). To overcome these issues, a new proximal sensor device has been developed to analyze multispectral reads in maize under stress conditions (*Spišić et al. 2022*), representing the first real high-troughput phenotyping research in maize plants in Croatia (*Šimić et al. 2022*). The authors developed a low-cost proximal sensing node retrieving reads at six wavelengths in red, near-infrared, and infrared, intended to capture important plant reflectance indices and fluorescence signals. Measurements in barley, wheat, and maize were carried out in order to evaluate the functionallity of the newly developed multispectral sensor for the prediction of physiological status. Additional instruments used were: Photon System Instruments Plant Pen PRI measuring leaf reflectance in a closed chamber at 525 and 592 nm, Trimble GreenSeeker measuring reflectance at undisclosed wavelengths, retrieving normalized difference vegetation index (NDVI), and a handled fluorimeter Hansatech Handy PEA. There were very strong correlations between comparable reads of the newly developed multispectral sensor and the commercial multispectral sensor. Proximal sensing nodes combined with novel communication devices (Internet of Things), emerging phenotyping technologies and methods (high-throughput phenotyping), and data science frameworks (machine learning) show promise in the transition to Agriculture 4.0/5.0.

In conclusion, chlorophyll *a* fluorescence technique is a useful tool for studying the physiology of maize plants and can provide valuable information for improving the productivity and sustainability of maize crops. Researchers at the Agricultural Institute Osijek have made important contributions to the study of chlorophyll fluorescence in maize, and their work has the potential to improve our understanding of the factors that affect maize growth and development. The use of this technique in the study of maize hybrids under different environmental conditions has provided important insights into the photosynthetic properties of maize and the potential for improving crop efficiency. However, it is essential to understand the relationship between photosynthesis and stress in maize to develop effective strategies for improving important traits in maize breeding. Further research in this area will help us understand the full extent of its benefits and applications.

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Application of Chlorophyll Fluorescence in Fruit Science

Ines Mihaljević and Marija Viljevac Vuletić

In orchards, fruit trees are exposed to a variety of abiotic stress factors that negatively affect their development, physiology, yield, and quality. Abiotic stressors such as high temperatures, UV light, drought, and salinity, can negatively affect photosynthesis by reducing photosynthetic efficiency, damaging chlorophyll pigments, and disrupting biosynthetic pathways, leading to changes in the morphology, physiology, and productivity of higher plants (*Muhammad et al. 2021*). As perennial woody plants, fruit trees exhibit more complex photosynthetic reactions to stress than herbaceous, depending on their age, species, and cultivar characteristics, as well as their capacity to overcome and adapt to stress conditions (*Swoczyna et al. 2022*). One of the most sensitive components of the photosynthetic apparatus, the photosystem II (PSII), is primarily affected by those stressors (*Gururani et al. 2015*). Therefore, knowing and understanding the photochemistry of stressed plants is one of the strategies to minimize the negative effects of these stresses. In recent years, different technics based on the measurement of chlorophyll fluorescence have been widely used to assess the impact of various stress factors on photosynthetic performance in many plant species, including fruit trees. Changes in fluorescence intensity at different stages of the photosynthetic process provide information on the efficiency of the photosynthetic apparatus, the rate of electron transport, the state of photochemical reaction centers, antennae, and the donor and acceptor sides of PSII (*Strasser et al. 2004*). The wide range of research on chlorophyll fluorescence demonstrates its usefulness in many areas of plant biology, agronomy, and ecology research. Although the measurement of chlorophyll fluorescence in fruit trees has become popular for monitoring the physiological responses of fruit trees to various stresses, research on fruit trees is still much less common compared to herbaceous plants. These studies can offer useful insights into the physiological condition of fruit trees, which can be used to increase productivity and quality in fruit production. Therefore, besides its usefulness in stress research, we will also present a variety of applications of chlorophyll fluorescence in the other aspects of fruit growing.

At Agricultural Institute Osijek, the Department for fruit growing, in cooperation with the Department of Agrochemical laboratory, started with photosynthesis research in 2010 with a doctoral dissertation on the photosynthetic performance of sour cherry genotypes under drought (*Viljevac 2012*). The implementation of chlorophyll fluorescence in fruit growing at the Agricultural Institute Osijek was continued by Ines Mihaljević through her dissertation on the effect of elevated temperature and excess light on apple genotypes *in vivo* (*Mihaljević 2016*). In the meantime and after the dissertations, both of them successfully implemented the technique in their research work, connecting the obtained results with other physiological and biochemical parameters and nutritional and pomological characteristics of the fruits, which is reflected in numerous published scientific works.

Apples are the most widely grown and economically important continental fruit in the world (*Harris et al. 2002*), and therefore the most investigated fruit specie regarding abiotic stress impact on growth, productivity, and fruit quality. High summer temperatures and high solar radiation can have a negative impact on the photosynthesis of apple trees and reduce the efficiency of photosynthesis (*Chen and Cheng 2009, Mupambi et al. 2018*). A study by *Duan et al. (2015)* used chlorophyll fluorescence as a non-invasive measurement to evaluate heat stress in apple trees and to reveal diurnal and seasonal changes in photosynthetic response. They monitored photosynthetic efficiency during summer months by measuring chlorophyll fluorescence and found that apple trees improve their thermotolerance during seasonal shifts by increasing both PSII and PSI activity. Using OJIP analysis, the authors demonstrated that heat stress causes perturbations in oxygen-evolving complex (OEC), reaction centers (RCs), and electron transport to the end of electron acceptors PSI, and that apple leaves examined at the end of the growing season were less susceptible to heat stress than young leaves. A significant reduction in photosynthetic capacity was detected in the leaves of two apple cultivars grown in the orchard under the influence of higher temperatures and excessive midday irradiance (*Mihaljević et al. 2020*). Reduction in PSII functionality of these cultivars was also observed due to damage to the OEC, where the parameter maximum quantum yield of PSII (F_v/F_m) clearly indicates the effects of stress. In the apple leaves, heat stress caused a significant decrease in the quantum efficiency of PSII electron transport, which was attributed to the reduction in photochemical quenching and the proportion of open PSII reaction centers (*Greer 2015*). Also, the JIP test showed to be efficient for the evaluation of physiological responses to short-term heat stress in the leaves of plum cultivars (*Viljevac Vuletić et al. 2022b*). They found that elevated temperature caused heat stress in two plum cultivars, seen as a decrease in water content, as an indicator of water-related stress, and a decrease in performance indexes (PI_{ABS} and PI_{total}) and the maximum quantum yield of PSII (F_v/F_m), with significantly pronounced changes found in modern cultivar Toptaste compared to traditional cultivar Bistrica. A decrease of named JIP parameters was pronounced in cultivar Toptaste because of increased minimal fluorescence (F_0) and absorption (ABS/RC), as well as an increase of Chl *b* in total chlorophylls. Additionally, the inactivation of RCs (RC/ABS) suggests that excitation energy was not trapped efficiently in the electron chain transport, which resulted in stronger dissipation (DI_0/RC) and the formation of ROSs. However, the use of chlorophyll *a* fluorescence as an indicator of the short-term temperature stress in the peach leaves was confirmed by *Martinazzo et al. (2012)*. Therefore, chlorophyll fluorescence and JIP test can be used as a basis in further plum breeding programs as a technics for quick screening of genotype tolerance for high-temperature stress.

In these times of disturbing climatic changes, shifts in temperature regimes during the vegetation season have a significant impact on fruit production. These changes can be manifested as low temperatures that occur in late spring when the fruit trees have already blossomed. *Vosnjak et al. (2021)* used chlorophyll fluorescence to evaluate the impact of low, non-freezing temperatures on the physiological status of sweet cherry trees and found a decrease in the maximum and effective quantum yield of PSII in all investigated cultivars, but the effect of chilling stress was more pronounced at lower temperatures, as well as in trees chilled for several consecutive nights over a single treatment.

Because the measurement of chlorophyll fluorescence provides a non-invasive and rapid assessment of plant performance, it is very commonly used to phenotype photosynthetic efficiency under different environmental conditions for testing genotypic differences in many plant species (*Banks 2018, Galicia-Juárez et al. 2021, Markulj Kulundžić et al. 2022*). By calculating the JIP test parameters, researchers can gain insight into the mechanisms of plant stress and adaptation and identify plants that are more tolerant to environmental stress. Overall, screening for drought and heat stress tolerance is a very important component of breeding programs aimed at developing fruit trees that can withstand environmental conditions and improve fruit yield under stress conditions. Most fruit species and cultivars differ in their ability to withstand high temperatures and excess light, including apples (*Li and Cheng 2011*). In the ecological environment of eastern Croatia, five apple cultivars were tested for their resistance to heat stress during three growing seasons (*Mihaljević et al. 2017*). The study found that different apple cultivars have different tolerance to high temperatures and high light stress, and that chlorophyll fluorescence can be a useful tool for evaluating these differences. According to the parameters (F_v/F_m) and photosynthetic performance (PI_{ABS}) determined during the study, Gold Rush was the cultivar with the best tolerance to higher temperatures and excess light.

The selection of drought-tolerant genotypes is essential for developing sustainable fruit production in today's climate-change conditions, which is why chlorophyll fluorescence is a powerful tool for the evaluation of drought stress extent on plants and the usage of obtained information to select drought-tolerant cultivars. Drought stress decreases the photosynthetic efficiency of fruit trees, as evidenced by a decrease in the maximum quantum yield of photosystem II (PSII) (*Ma et al. 2015, Wang et al. 2018*). *Mihaljević et al. (2021a)* evaluated traditional and modern apple cultivars under drought conditions and revealed a significant increase in absorption per active reaction center (ABS/RC), trapped energy flux per active reaction center (TR_0/RC), electron transport flux per active reaction center (ET_0/RC), and dissipation energy per active reaction center (DI_0/RC), indicating inactivation of PSII reaction centers. They found higher positive L and K bands in more susceptible cultivars with lower photosynthetic efficiency, suggesting disturbances in energetic connectivity between PSII units and in the oxygen-evolving complex on the donor side of PSII. Furthermore, in another study, *Mihaljević et al. (2021b)* used chlorophyll fluorescence to identify a sweet cherry cultivar with improved photosynthetic efficiency under drought conditions. They reported that drought stress caused a greater decline in performance indices (PI_{ABS} and PI_{total}) and damage to PSII oxygen-evolving complex in a modern cultivar, in contrast to an autochthonous cultivar that showed an advantage over the modern cultivar in PSII efficiency under progressive drought.

Viljevac Vuletić et al. (2022a) successfully revealed photosynthetic variability of sour cherry ecotypes from a phenotypic heterogenic population of cultivar Oblačinska sour cherry, under drought conditions using the chlorophyll fluorescence technique. ChlF highlighted PI_{ABS}, PI_{total}, and the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (ψE_0) as the most sensitive and, thus, the most informative JIP parameters for drought screening. Also, chlorophyll fluorescence differentiates sour cherry cultivar differences under drought (*Viljevac et al. 2013*). Confrontation of two sour cheery cultivars OS and Kelleris 16 under drought revealed down-regulation of photosynthesis in drought-treated Kelleris 16. Despite the unchanged maximum quantum yield of PSII in drought-treated leaves of genotype OS, overall photosynthetic performance expressed as PI_{ABS} was down-regulated in both investigated genotypes. However, the decrement of PI_{ABS} was much more pronounced in the genotype Kelleris 16, mainly because of changes in a certain fraction of RCs, which became dissipative centers, in order to avoid photooxidative damage to photosynthetic apparatus. Also, electron transport was impaired, which led to impaired CO_2 fixation and photosynthesis.

In this era of climate change, altogether with drought, high temperature and solar radiation, waterlogging has also been one of the significant problems in plant cultivation worldwide. *Bhusal et al. (2020)* exposed two apple cultivars, grown as bi-leader trees, to long-term waterlogging conditions and found a significant decrease in photosynthetic efficiency seen as a decrease in the maximum quantum yield of PSII (F_v/F_m) in both investigated apple cultivars. Furthermore, *Xu et al. (2022a)* successfully used chlorophyll fluorescence to evaluate waterlogging tolerance of different peach rootstocks and found that the highest waterlogging tolerance in the Hossu genotype was based on better light energy usage efficiency and efficient dissipation of absorbed excess light energy.

Besides heat and high light stress, in the summer months, fruits may experience a physiological disorder called sunburn, which causes significant economic losses in fruit production (*Racskó and Schrader 2012*, *Xu et al. 2022b*). Although fruit growers often take agrotechnical measures to reduce the risk of sunburn, these actions are not always effective, especially during extreme weather conditions, where sunburn can still occur. *Blackhall et al. (2020)* used the JIP test to investigate changes in the PSII photochemical process in apple peels under high solar radiation to understand the susceptibility of apple peels to photooxidative damage. They found that fruits not exposed to sunlight during growth and development may be more susceptible to photooxidative damage when suddenly exposed to sunlight during early and mid-development, which could help develop strategies to reduce sunburn risk and minimize its impact on apple production. By monitoring the chlorophyll fluorescence of apple trees, growers can early identify potential problems such as sunburn stress, reduce the risk of sunburns, and take steps to mitigate the negative effects, such as providing shade (*Sotiropoulos et al. 2016*) or applying foliar sprays such as kaolin clay (*Sarooghinia et al. 2020*). Kaolin clay, as natural clay, was found to be an effective tool to mitigate plant stress and improve yield (*Wand et al. 2006, Brito et al. 2019). Mihaljević et al. (2022)* used the chlorophyll fluorescence method to evaluate physiological changes in pear leaves treated with kaolin particle film sprays and showed that kaolin sprays improved photosynthetic efficiency and antioxidant activity of pear leaves under higher temperatures and excess light conditions. These results suggest that kaolin particle spraying may be an effective tool for mitigating heat and high light stress in pear and apple production.

Monitoring the health and vigor of fruit trees is a key element for successful fruit production. Therefore, it is very important to intervene on time to prevent further damage or the spread of diseases in orchards. By monitoring chlorophyll fluorescence, growers can detect early signs of stress or disease and, along with proper irrigation and plant nutrition, ensure or promote healthy growth and higher yields. Chlorophyll fluorescence parameters can be used to check for disease occurrence in fruit trees, where changes in fluorescence parameters have been shown to be effective indicators of the presence and severity of various diseases. The study performed by *He et al. (2022)* found the important role of photosynthesis in apple resistance to apple ring rot. They showed that apple resistance to apple ring rot was associated with photosynthesis and that chlorophyll fluorescence declined with declining apple ring rot resistance. *Mendes et al. (2020)* has shown that changes in chlorophyll fluorescence parameters can be symptomatic of the presence of *Erwinia amylovora* diseases in apple trees, where the maximum quantum yield (F_v/F_m) significantly decreased in infected trees. The technique of chlorophyll fluorescence was used to detect pathogens and determine the nutritional and health status of apple trees (*Spáčilová and Šafránková 2011*).

The fruit ripening process is accompanied by changes in the chlorophyll content and composition (*Park et al. 2018*). These changes can affect the photosynthetic apparatus, leading to a decline in chlorophyll fluorescence as the fruit ripens (*Song et al. 1997*). Therefore, changes in chlorophyll fluorescence parameters can be used to predict damage to fruits during storage. Physiological changes in pears and apples during a shelf-life trial were studied and found that chlorophyll fluorescence can be used to predict shelf-life quality (*Huybrechts and Valcke 2005*). This method allows a quick quality assessment of the stored fruit to predict the changes in physical characteristics during storage (*Zsom et al. 2016*). Chlorophyll fluorescence has been effectively used in earlier studies to diagnose and analyze fruit disorders like browning in pears, suggesting that it is a promising tool to detect browning injuries in pears before they occur (*Saquet and Streif 2002, Gardé et al. 2012, Guerra et al. 2012*). In this way, the occurrence of browning damage can be indicated, which could be helpful as preventive measures during storage, such as adjusting the storage temperature and humidity to extend the shelf-life of the fruit.

Numerous applications of chlorophyll fluorescence in fruit production were evident through scientific papers. *Li et al. (2021)* investigated the effects of herbicide drift on various types of fruit trees (apple, peach, and grape) using chlorophyll fluorescence measurement of the leaves after the application of glyphosate (systemic herbicide) and GLA (a non-selective, contact non-conducting herbicide). Results revealed a decrease in PSII activity after glyphosate and GLA application in three fruit trees. All species were less susceptible to glyphosate and more susceptible to GLA (the action modes of the two herbicides differ) and neither fruit tree recovered after glyphosate spraying, but apple and peach (not grape) recovered after GLA spraying, which is valuable information for fruit growers to reduce herbicide use or cover their fruit trees while spraying. Another successful usage of chlorophyll fluorescence is in the impact assessment of different light filters for rain-shelter cultivation of fruit on leaf photosynthetic features and fruit quality. Therefore, *Zhang et al. (2018)* studied chlorophyll fluorescence characteristics in a late-maturing peach variety under five different filters compared to natural light and found that a neutral filter in a rain-shelter preserved high photosynthetic capacity and even improved fruit quality. Besides cultivation under rain-shelters, a significant impact on fruit yield and quality relies on tree shape in the orchard. Different shapes of pear trees were evaluated to assess the impact on photosynthesis and fruit quality because the level of photosynthetically active radiation (PAR) varies by tree shape (*Zhao et al. 2022*). Chlorophyll fluorescence parameters in this research showed that single- and double-arm tree shapes are well-ventilated and light-transmitting, which promotes fruit growth and quality. A much different application of chlorophyll fluorescence can be found in the paper of *Mitrofanova et al. (2021).* They used chlorophyll fluorescence to evaluate the physiological state through the photosynthetic activity of micropropagated plantlets from hybrid embryos originating from four peach cross combinations in order to create new economically valuable cultivars.

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Part 4

Case Study

Breeding of Winter Wheat and Barley with the Help of the Photosynthetic Parameters

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Introduction

The global warming of our planet, which causes significant climate changes, has been a multidisciplinary topic for decades with the target of preserving and improving life on Earth. In this, the safety of quality food production in sufficient quantities and healthy environment preservation is imperative (*Reynolds et al. 2016*), in which plant breeding with appropriate agrotechnical measures plays a significant role. Therefore, according to recent research, supporting tools for plant breeding are enzymes and hormones of antioxidative stress (*Antunović Dunić et al. 2015, Vuković et al. 2022, Duvnjak et al. 2023, Dvojković et al. 2023*) and parameters calculated from chlorophyll *a* fluorescence (JIP test) (*Kovačević et al. 2013, Antunović Dunić et al. 2015, Kovačević et al. 2015, Kovačević et al. 2017, VIljevac Vuletić and Španić 2020, Katanić et al. 2021, Španić et al. 2021, Španić et al. 2022*), especially the performance index (PI_{ABS}). It is necessary to highlight the significant works of the authors: *Schreiber et al. 1994, Strasser et al. 1995, Strasser et al. 2000, Strasser et al. 2004, Oukarroum et al. 2007, Lepeduš et al. 2009, Kalaji et al. 2012* in the methods of determining parameters based on chlorophyll *a* fluorescence (JIP test) and the possibilities of their application.

Genotypes (varieties) of wheat and barley show various tolerances when they are exposed to different stress conditions. Stress tolerances are quantitative traits such as yield and quality (*Araus et al. 1998, Guóth et al. 2009, Španić et al. 2013, Kovačević et al. 2017, Öztürk and Aydin 2017*). Investigations of the interaction among genotypes and environments give useful information concerning the stability of yield and its quality in wheat and barley (*Blum 1989, Ceccarelli et al. 2000, Lalić et al. 2009*), and by analysing these data, we can learn about the reaction of different genotypes to different stress conditions. In this theme, the attention is on research in the breeding and genetics of wheat and barley, although work is also done on other species of agricultural plants, which has been carried out at the Agricultural Institute Osijek for decades through the cooperation of scientists: plant breeders, agricultural technicians, biologists, and food technologists.

Grain yield is a particularly complex trait because its expression is greatly influenced by genetic inheritance, environmental conditions such as climate, weather conditions, diseases, pests, and agrotechnics. However, the relationship between photosynthetic parameters and grain yield of different wheat and barley genotypes can be highly variable (*Araus et al. 1998, Guóth et al. 2009, Španić et al. 2013, Kovačević et al. 2017, Öztürk and Aydin 2017*).

Since it is usually accepted that the duration and efficiency of photosynthesis, particularly in the flag leaf, has a significant effect on grain yield (*Guóth et al. 2009, Kenny 2016, Španić et al. 2022*), physiologists and plant breeders worldwide tend to develop indirect and non-invasive methods for estimating relationships between physiological properties and economically valuable agronomic traits of winter wheat and barley genotypes (*Araus et al. 1998, Long et al. 2006, Aliyev 2012, Kovačević et al. 2011, Kalaji et al. 2012, Kovačević et al. 2013, Španić et al. 2013, Kovačević et al. 2015, Reynolds and Langridge 2016, Kovačević et al. 2017, Viljevac Vuletić and Španić 2020, Katanić et al. 2021, Španić et al. 2022, Duvnjak et al. 2023, Dvojković et al. 2023*). The breeding for the yield and components of yield when complemented by the data on the physiological mechanisms of wheat became a logical choice for this task (*Apel and Hirt 2004, Dhanda et al. 2004, Shao et al. 2005, Zhu et al. 2010*).

Many researchers prefer PI_{ABS} (photosynthetic performance index) to evaluate the response of different species and genotypes of agricultural plants to environmental stress, such as drought, high air and soil temperature, and other stresses (acidity, salinity, mobile aluminium, diseases, light stress, and other unfavourable influences) (*Strasser et al. 2004, Oukarroum et al. 2007, Kalaji et al. 2012*). The most frequently investigated photosynthetic efficiency parameters are the maximum quantum yield of PSII (TR_0/ABS or F_v/F_m) and performance index for energy conservation from exciton to the reduction of intersystem electron acceptors (PI_{ABS}) (sometimes quantum yield of electron transport, i.e. ET_0/ABS), in particular their relationship to the agronomic traits of different species of cultivated plants and genotypes under different stress conditions, stages of growth, and environments (*Araus et al. 1998, Strasser et al. 2004, Oukarroum et al. 2007, Araus et al. 2008, Guóth et al. 2009, Balouchi 2011, Gholamin and Khayatnezhad 2011, Kovačević et al. 2011, Akhkha et al. 2013, Kovačević et al. 2013, Španić et al.*

2013, Czyczylo-Mysza et al. 2013, Kovačević et al. 2015, Kovačević et al. 2017, Španić et al. 2022).

Th*e rht* genetic complex (genes that reduce height) is very important from the aspect of wheat breeding as well as from the physiological and agronomic aspects (*Gent and Kiyomoto 1997, Worland et al. 1998*). These genes modulate the morphology and physiology of wheat, decreasing the leaf area but increasing photosynthetic efficiency per leaf area unit. The importance of Rht, Vrn, Ppd, and Eps genes as markers in physiological breeding for the targeted improvement of wheat agronomic properties such as grain yield and adaptability reactions is being investigated by *Reynolds et al. (2009)*, as well as Ppd and Vrn phenological genes, complex growth genes (Rht) and aluminum tolerance gene TaALMT in wheat genotypes by *Eagles et al. (2014*).

A comparative study of the genetic diversity in the population of 89 Croatian (released between 1936 and 2006) and 523 winter wheat genotypes of European origin (Denmark, France, Germany, Hungary, Sweden, and the United Kingdom) estimated the highest genetic similarities of Croatian genotypes with Hungarian, which are regionally (geographically) close (*Novoselović et al. 2016*). Recognizable properties of Croatian winter wheat genotypes are a short stem, which is mainly controlled by the Rht major genetic complex, i.e. the most prominent Croatian genotypes have a dwarf and semi-dwarf habit, usually a shorter vegetation period, i.e. earlier heading (*Novoselović et al. 2016*) and their specific reaction to a shortened photoperiod (*Martinić-Jerčić 1975*) is seen as good adaptability even in conditions of a shorter day.

Case Study: Winter Wheat

Fourteen winter wheat genotypes were grouped into two groups of seven genotypes each according to stem height (dwarf – D, and semi-dwarf – SD). In the group semi-dwarf (SD) were genotypes: Antonija, Rebeka, Vulkan, Žitarka, Aphache, Grandior, and Renan, while in the group dwarf (D) were genotypes: Alka, Katarina, Kraljica, Leuta, Lucija, Renata, and Srpanjka. Most of the listed varieties are creations of the Agricultural Institute Osijek in the period from 1985 to 2011, and the genotypes Renan (France – INRA), Apache (France – NIF), and Grandior (Austria – RWA) are introductions. In both genotype groups (D and SD), there are two older genotypes that had the largest share in wheat production in Croatia for more than 20 years, Žitarka (1989–2000) and Srpanjka (2001–2014). The long-term presence of some genotypes in wide wheat production in Croatia indicates possession of some other attributes, such as quality of dough and bread coupled with grain yield stability (*Španić et al. 2016*). The relationship of photosynthetic parameters with stem height, components of grain yield, and grain yield was investigated by combining experiments in vegetation pots and experiments in the field.

Figure 1. Comparative field trials with different winter wheat genotypes at the stage after heading and grain formation (left) and harvest of experimental plots with a combine harvester (right) (photo: Kovačević J, 2011).

The multi-environmental trials were carried out by the randomized block design (RBD) in four repetitions of the basic plots with a net area of 7.56 m^2 (1.08 × 7.00). The sowing time of the trials was in the autumn of 2011 and 2012, and the time of harvest was at the beginning of July 2012 and 2013. The density of sowing was 600 germinated seeds m^{-2}. The locations of the multi-environmental trials were in the eastern part of Croatia (Osijek). Grain yield, stem height, and mass of 1000 grains were the traits observed in the multi-environmental trials (**Figure 1**). Other traits (water use – WU, water use efficiency – WUE, biomass weight, number of ears per pot, and kernels number per ears), as well as chlorophyll *a* fluorescence parameters (ChlF), were analysed in vegetative pots (**Figure 2**). The mentioned genotypes (varieties) of winter wheat are known for wheat production in many countries of Central and Southeastern Europe and have already been tested under the conditions of multi-environmental field trials (*Drezner et al. 1999, Španić et al. 2011*).

Plants of 14 winter wheat genotypes were grown in vegetative pots according to the two-factorial experimental design (WW – well watered; WS – moderate short-term drought stress) with three replications. Pots were filled with the upper layer (depth up to 30 cm) of soil from the experimental field of the Agricultural Institute Osijek in the Republic of Croatia (45°32″N, 18°44″E) and saturated with water to 39% volume of soil (100% FC – field capacity or water holding capacity). The soil in every pot had good fertility and the same mechanical, physical, and chemical composition. The pore volume of the soil was 49%, retention water capacity 39%, and air capacity 10%. Soil volume was 8,600 cm^3 per vegetative pot, and it was measured ten days after filling and saturation of soil with water. Sowing density in the pot experiment was calculated to match the field sowing density of 450 seeds per m^2, resulting in sowing 24 seeds per pot arranged in a circle with equal spacing between seeds and from the edge of the vegetative pot, giving the distance of 5 cm between the seed (**Figure 2**).

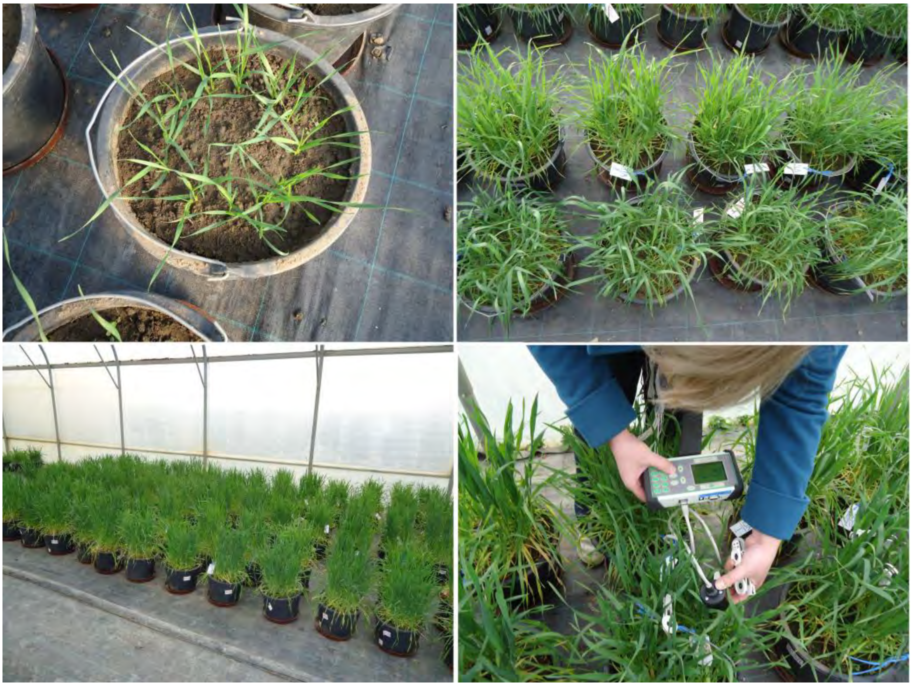

Figure 2. The winter wheat and winter barley experiments in vegetative pots (photo: Kovačević J, 2011).

Sowing was done seven days after soil water saturation on 18th December 2012. The germination was observed on January 5th, 2013. During the winter months (January, February, and two decades of March), in the greenhouse where the research was conducted, daily air temperature varied between 11 and 18 °C, and night temperature between 2 and 8 °C, providing proper conditions for the vernalisation of all studied genotypes. During the same period of time, the relative air humidity varied in range from 92 to 99%. At the beginning of spring, at the stem elongation stage of winter wheat growth, the daily air temperature varied between 19 and 27 °C, and the night temperature between 7 and 13 °C, while the relative air humidity varied, ranging from 71 to 92%. In the period from the flag leaf stage to the beginning of grain development, the daily air temperature varied between 25 and 32 °C, and the night temperature between 12 and 18 °C with the relative air humidity from 48 to 72%. The maximum daily temperature during the grain development (filling) to maturity was 38 °C, and the minimum air humidity was 36%.

Water treatments were:

WW – well-watered: the soil water content in pots (75–85% FC) – water content in the pores of soil 29–33% volume of soil; air content in the pores of soil 16–20% volume of soil.

WS – moderate short-term drought stress: the soil water content in pots (45–50% FC) – water content in the pores of soil 18–20% volume of soil (near wilting point); air content in the pores of soil 29–31% volume of soil.

The soil water content in the vegetative pots was measured and calculated every day as the difference between water content at 100% of FC (39% volume of soil) and soil water reduction in each vegetative pot of both water treatments (WW and WS). During the measuring of the ChlF, the water content in the soil in the WS treatment was for a short time (24 hours) at the wilting point, while during the rest of the time, it was at the optimal level. In the WW treatment, the water content was always at the optimal level. The same method in vegetation pots as in field comparative experiments was used in experiments with winter barley.

The measuring of chlorophyll *a* fluorescence (ChlF) was conducted at six points after water treatments. The growth and developmental stages of wheat plants at the time points of water treatments and measurements are given in **Table 1**. The ChlF measurements were performed in the morning hours from 9:00 to 10:00 h for stages a, b, c, and d, and between 8:00 and 9:00 h for the e and f stages of growth. The ChlF was measured when the soil water contents were at the wilting point in WS treatment and at the optimal level in WW treatment. The measurements of ChlF were carried out on the second fully developed leaf from the top of the plant in the variants of experiment WWa, WSa, WWb, WSb, WWc, WSc, WWd, and WSd and on the flag leaves in the WWe, WSe, WWf, and WSf treatments on randomly chosen three plants per pot in three replicates, making 9 measurements per genotype for each treatment. A total of 1,512 plants and 252 plants for both treatments in each measurement of the experiment were chosen for the measurement of the ChlF by portable fluorimeter Handy Plant Efficiency Analyser (Handy PEA, Hansatech Instruments Limited, King's Lynn, UK). JIP test was used to calculate photosynthetic parameters: TR_0/ABS, ET_0/ABS, the probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (ET_0/TR_0), absorption flux (of antenna chlorophylls) per active RC (ABS/RC), trapping flux (leading to Q_A reduction) per active RC (TR_0/RC), electron transport flux (further than Q_A^-) per active RC (ET_0/RC), dissipated energy flux per active RC (DI_0/RC), absorbed excitation energy per unit cross section (ABS/CS_0), trapping per excited cross section (TR_o/CS_0), electron transport per excited cross section (ET_0/CS_0), dissipation per excited cross section (DI_0/CS_0), the density of active RC per excited cross section (RC/CS_0), the density of RC on chlorophyll *a* basis (RC/ABS), flux ratio trapping per dissipation (TR_0/DI_0), electron transport further than primary acceptor Q_A ($ET_0/(TR_0\text{-}ET_0)$), and PI_{ABS} (*Strasser et al. 2000, Lepeduš et al. 2009*).

Table 1. Measurement points and description of growth and organogenesis stages of winter wheat.

Measurement	Date of ChlF measurement	Stage of growth (*Zadoks et al. 1974*)	Stage of organogenesis (*Kuperman 1965*)
a	March 13th	Tillering	II
b	March 18th	Tillering	II and III
c	March 20th	Tillering	II and III
d	April 8th	Beginning of stem elongation or jointing (appearance of flag leaf in early genotypes)	IV and V
e	April 15th	Flag leaf or booting	V and VI
f	April 26th	Heading	VIII

The photosynthetic parameters, heading time, the number of spikes, and leaf senescence were observed in the vegetative pots. The leaf senescence was estimated visually at the flag leaf stage (1 – the absence of senescence; 9 – maximum senescence) because, at the early stage of wheat growth, the leaf senescence is absent in all studied winter wheat genotypes.

Two genotype groups of stem height (D and SD), which were significantly different ($P \leq 0.01$) (**Table 2**), were significantly different ($P \leq 0.01$) for PI_{ABS} at the tillering stage (a, b, c – Zadoks scale 26–29) (**Table 5**) in all combinations (D vs. SD; WW vs. WS inside D and SD groups). PI_{ABS} values were higher in WS treatment compared to WW treatment in all three ChlF measurements at the tillering growth stages (a, b, c), which corroborates with previous results with wheat and barley (*Kovačević et al. 2013, Kovačević et al. 2015, Kovačević et al. 2017*). PI_{ABS} values were lower in semi-dwarf (SD) compared to dwarf (D) habitus (**Table 5**), which was also shown at later stages of wheat growth (d, e, f) (**Table 6**), and this agrees with other research (*Gent and Kiyomoto 1997, Balouchi 2011, Kovačević et al. 2017*).

At later stages of growth (d, e, f – Zadoks scale 35–60), significant differences in PI_{ABS} were estimated between water treatments (WW vs. WS) in both D and SD wheat genotype groups only at the stage of the beginning of heading (f) with lower values in WS treatment (**Table 6**). The two groups of the winter wheat genotypes (D and SD) were significantly different ($P \leq 0.01$) in the PI_{ABS} parameter (**Tables 5** and **6**), ET_0/ABS, ET_0/CS_0, ET_0/TR_0, and $ET_0/(TR_0\text{-}ET_0)$ (**Tables 7** and **9**) in all three measurements during the tillering stages (a, b, and c). The group of wheat genotypes with a shorter stem (D) and higher values of the mentioned photosynthetic parameters achieved a lower grain yield in multi-environment field experiment than the group of genotypes with a longer stem

(SD) (**Table 2**). Correlation coefficients between PI_{ABS} and grain yield, which were negative, confirmed the stated claim (**Table 14**).

Group D had a lower mass of 1000 grains, a higher number of spikes per pot, earlier heading, and lower values of leaf senescence than the SD group of genotypes (**Tables 3** and **4**). Also, it had significantly higher values of PI_{ABS}, ET_0/ABS, ET_0/TR_0, $ET_0/(TR_0\text{-}ET_0)$, and DI_0/RC. Photosynthetic parameters ET_0/ABS, ET_0/TR_0, $ET_0/(TR_0\text{-}ET_0)$ were in strong positive correlation ($P\leq0.001$) with PI_{ABS}, while DI_0/RC was the opposite, i.e. in strong negative correlation ($P\leq0.001$) with PI_{ABS}, which was estimated in all ChlF measurements (a, b, c, d, e, and f) in both water treatments (WW and WS) (**Table 11**). This suggests that the PI_{ABS} is the most informative parameter, as it was found earlier in similar research (*Strasser et al. 2004, Oukarroum et al. 2007*).

Table 2. Stem height and grain yield of winter wheat genotypes grouped into two groups on the basis of stem height (D and SD) in the field trials in Osijek (east part of Croatia) harvested in 2012 and 2013.

Group	Genotype	Stem height, cm			Grain yield, t ha^{-1}		
		2012	2013	Average	2012	2013	Average
Dwarf (D)	Srpanjka	53.0	62.0	57.5	7.945	5.539	6.742
	Renata	57.0	65.0	61.0	8.107	6.462	7.285
	Katarina	61.0	64.0	62.5	9.601	7.157	8.379
	Leuta	61.0	67.0	64.0	10.046	6.500	8.273
	Lucija	61.0	72.0	66.5	7.747	5.980	6.863
	Kraljica	63.0	73.0	68.0	9.195	7.999	8.597
	Alka	67.0	75.0	71.0	9.624	7.214	8.419
	Average D	60.4	68.3	64.4	8.895	6.693	7.794
Semi-dwarf (SD)	Antonija	66.0	77.0	71.5	10.223	8.744	9.483
	Žitarka	67.0	86.0	76.5	7.831	6.884	7.357
	Vulkan	76.0	84.0	80.0	10.158	7.165	8.661
	Apache	72.0	91.0	81.5	9.743	10.372	10.057
	Renan	77.0	90.0	83.5	9.019	7.462	8.240
	Grandior	75.0	100.0	87.5	10.512	9.909	10.210
	Rebeka	84.0	95.0	89.5	10.143	7.206	8.675
	Average SD	73.9	89.0	81.4	9.661	8.249	8.955
	Average	67.1	78.6	72.9	9.278	7.471	8.374
	LSD $_{0.01}$	6.32	7.98	5.73	1.026	1.390	1.096
	D vs. SD	**	**	**	**	**	**

$LSD_{0.01}$ – The test of the significance between two average values of the genotypes at $P\leq0.01$.

*** Differences between two average values of the groups of the genotypes (D vs. SD) are significant at $P\leq0.01$; ns – not significant*

Table 3. The thousand grains mass of the winter wheat genotypes examined in the field trials in Osijek in 2012 and 2013 and spike number per vegetative pot.

Group	Genotype	1000 grains mass, g			Spike number per vegetative pot		
		Osijek 2012	Osijek 2013	Average	WW	WS	Average
Dwarf (D)	Srpanjka	33.34	23.48	28.41	53.0	39.3	46.2
	Renata	41.75	30.43	36.09	36.3	32.7	34.5
	Katarina	36.60	28.23	32.41	36.0	31.0	33.5
	Leuta	40.73	27.35	34.04	45.3	41.0	43.2
	Lucija	35.04	24.85	29.94	42.0	38.7	40.3
	Kraljica	37.24	28.75	33.00	39.7	33.3	36.5
	Alka	36.56	29.40	32.98	48.3	42.3	45.3
	Average D	37.32	27.50	32.41	43.0	36.9	39.9
Semi-dwarf (SD)	Antonija	37.02	30.90	33.96	44.3	46.0	45.2
	Žitarka	44.10	34.53	39.31	32.7	27.7	30.2
	Vulkan	36.33	33.30	34.81	43.7	45.3	44.5
	Apache	42.68	40.63	41.65	39.0	35.3	37.2
	Renan	48.83	40.10	44.46	28.7	33.7	31.2
	Grandior	43.66	38.40	41.03	36.0	32.7	34.3
	Rebeka	50.89	44.45	47.67	26.0	26.3	26.2
	Average SD	43.36	37.47	40.41	35.8	35.3	35.5
	Average	40.34	32.49	36.41	39.4	36.1	37.7
	LSD $_{0.01}$	2.49	3.35	2.48	7.12	8.54	7.84
	D vs. SD	**	**	**	**	ns	**

LSD$_{0.01}$ – The test of the significance between two average values of the genotypes at P≤0.01.

*** Differences between two average values of the groups of the genotypes(D vs. SD) are significant at P≤0.01; ns – not significant.*

Table 4. The time of heading (ear emergence) and leaf senescence evaluation of the winter wheat genotypes in the vegetative pot trial.

Group	Genotype	Time of heading, days in April			Leaf senescence: 1- absent; 9 - full		
		WW	WS	Average	WW	WS	Average
Dwarf (D)	Srpanjka	20	20	20	2.0	3.3	2.7
	Renata	23	23	23	2.0	3.3	2.7
	Katarina	23	23	23	2.0	3.0	2.5
	Leuta	23	22	23	2.0	3.0	2.5
	Lucija	19	19	19	2.3	3.3	2.8
	Kraljica	24	24	24	2.0	3.3	2.7
	Alka	23	23	23	2.0	4.0	3.0
	Average D	22	22	22	2.0	3.3	2.7
Semi-dwarf (SD)	Antonija	24	22	23	2.0	4.0	3.0
	Žitarka	23	24	23	2.3	5.3	3.8
	Vulkan	23	22	23	2.7	4.0	3.3
	Apache	28	27	28	2.3	5.7	4.0
	Renan	31	31	31	2.0	5.0	3.5
	Grandior	27	26	26	2.0	4.3	3.2
	Rebeka	28	27	28	2.0	4.3	3.2
	Average SD	26	26	26	2.2	4.7	3.4
	LSD $_{0.01}$	1.6	2.5	2.2			
	D vs. SD	**	**	**			

LSD$_{0.01}$ – The test of the significance between two average values of the genotypes at P≤0.01.
*** Differences between two average values of the groups of the genotypes(D vs. SD) are significant at P≤0.01.*

Table 5. Photosynthetic performance index (PI_{ABS}) in the winter wheat genotypes and groups of genotypes (D and SD) in the well-watered (WW) and short-term drought stress (WS) treatments through the tillering stages of growth (treatments: WWa, WSa, WWb, WSb, WWc, WSc).

Group	Genotype	Photosynthetic performance index (PI_{ABS}) through the growth stages (tillering – a, b, c)					
		WWa	WSa	WWb	WSb	WWc	WSc
Dwarf (D)	Srpanjka	2.706	3.284	2.627	3.214	2.806	3.558
	Renata	2.805	2.896	2.090	3.027	2.467	2.881
	Katarina	2.132	2.685	2.342	2.798	2.380	3.045
	Leuta	2.564	2.700	2.512	3.214	2.662	2.968
	Lucija	2.611	2.955	2.465	3.164	2.236	3.182
	Kraljica	2.874	2.992	2.620	3.314	2.623	3.445
	Alka	3.015	2.939	2.777	3.518	2.806	3.372
	Average D	2.672	2.922	2.490	3.178	2.569	3.207
Semi-dwarf (SD)	Antonija	2.393	2.575	2.177	2.469	2.204	2.643
	Žitarka	2.579	3.009	2.344	2.539	2.226	2.594
	Vulkan	1.991	2.001	1.778	2.588	1.824	2.524
	Apache	2.313	2.816	2.259	2.741	2.139	2.629
	Renan	2.409	2.796	2.095	2.707	2.078	2.686
	Grandior	2.675	2.960	2.096	2.721	2.043	2.911
	Rebeka	2.617	2.744	2.202	2.501	2.130	2.775
	Average SD	2.425	2.700	2.136	2.610	2.092	2.680
	Average	2.549	2.811	2.313	2.894	2.331	2.944
	$LSD_{0.01}$	0.539	0.565	0.396	0.431	0.367	0.484
	Average: D vs. SD	**	**	**	**	**	**
	D: WW vs WS	**		**		**	
	SD: WW vs. WS	**		**		**	

$LSD_{0.01}$ – *The test of the significance between two average values of the genotypes at P≤0.01.*

** Differences *between two average values of the group of the genotypes are significant at P≤0.01.*

The study of photosynthetic parameters at the early stages (tillering) of wheat growth may be interesting from the breeding point of view because it is easy and cheap to carry out, and it is possible to investigate a large number of genotypes. Moreover, this is supported by fewer errors at the earlier stages of development (tillering stage) than at the flag leaf stage and heading time (Table 12), which was also suggested in a similar study (*Kovačević et al. 2017*). Also, positive correlation coefficients of the parameters PI_{ABS} with TR_0/ABS, ET_0/ABS, ET_0/TR_0, RC/ABS, and TR_0/DI_0 and negative correlation coefficients with ABS/RC, TR_0/RC, DI_0/RC, and DI_0/CS_0 in the continuity of all six measurements (a–f) in both treatments (WW and WS) justify the use of photosynthetic parameters obtained by ChlF method (**Table 11**). The potential number of spikes, spikelets per spike, and flowers in the spikelets, as key determinants of multiple grain yield components, are determined at the early developmental stages of winter wheat (a, b, c, d), so a dry season at the beginning of spring can significantly reduce grain yield of winter wheat. *Munns (2002)* and *Plaut (2003)* also pointed out that the tillering stage of growth is considered to be the critical period of wheat responses to soil water deficits. At later stages of wheat growth and development, the occurrence of leaf senescence and diseases may be the cause of a greater error variation (MSe) (**Table 12**). The different degree of leaf senescence has an impact on physiological properties because the decline of photosynthetic activity is caused by degradative processes (*Harding et al. 1990, Al-Khatib and Paulsen 1994*).

Table 6. Photosynthetic performance index (PI_{ABS}) in the winter wheat genotypes and groups of genotypes (D and SD) in the well-watered (WW) and short-term drought stress (WS) treatments through the stages of growth: beginning of stem elongation (treatments WWd, WSd), flag leaf development (WWe, WSe) and beginning of heading (WWf, WSf).

Group	Genotype	Photosynthetic performance index (PI_{ABS}) through the growth stages (stem elongation – d, flag leaf – e, and beginning of heading – f)					
		WWd	WSd	WWe	WSe	WWf	WSf
Dwarf (D)	Srpanjka	4.473	4.238	4.881	4.476	4.340	3.638
	Renata	4.278	4.500	4.195	4.922	4.770	4.286
	Katarina	3.961	4.368	4.324	4.266	4.357	3.480
	Leuta	4.065	4.704	4.279	4.100	3.905	3.670
	Lucija	3.060	3.570	4.138	4.492	3.740	3.272
	Kraljica	4.258	3.981	4.777	4.355	4.603	4.215
	Alka	4.164	4.176	4.063	3.641	4.142	3.108
	Average D	4.037	4.220	4.380	4.322	4.265	3.667
Semi-dwarf (SD)	Antonija	3.046	3.335	3.729	3.185	3.446	2.830
	Žitarka	3.243	3.338	4.162	3.429	4.062	2.468
	Vulkan	3.021	3.184	3.568	3.625	3.806	3.095
	Apache	3.612	3.464	3.857	3.902	3.967	2.517
	Renan	2.949	3.110	3.659	3.849	2.617	2.894
	Grandior	3.569	3.493	4.424	4.105	4.149	3.591
	Rebeka	3.139	3.723	4.022	4.457	3.619	3.734
	Average SD	3.226	3.378	3.917	3.793	3.667	3.018
	Average	3.632	3.799	4.149	4.058	3.966	3.343
	$LSD_{0.01}$	1.069	1.093	0.748	0.751	0.940	0.718
	Average: D vs. SD	**	**	**	**	**	**
	D: WW vs WS	ns		ns		**	
	SD: WW vs. WS	ns		ns		**	

$LSD_{0.01}$ – The test of the significance between two average values of the genotypes at P≤0.01.
*** Differences between two average values of the group of the genotypes are significant at P≤0.01; ns – not significant*

Table 7. The average values of photosynthetic parameters (TR$_0$/ABS, ET$_0$/ABS, ET$_0$/TR$_0$, ABS/RC, TR$_0$/RC, ET$_0$/RC, and DI$_0$/RC) of the groups of winter wheat genotypes (D and SD) in the well-watered (WW) and moderate short-term drought stress (WS) treatments and the average values of both treatments at the tillering stages of growth (WWa, WSa, WWb, WSb, WWc, WSc).

Group	Water treatment	Photosynthetic parameters					
		TR$_0$/ABS	ET$_0$/ABS	ET$_0$/TR$_0$	ABS/RC	TR$_0$/RC	DI$_0$/RC
D	WWa	0.828	0.461	0.556	2.313	1.914	0.399
	WSa	0.832	0.471	0.566	2.256	1.877	0.378
	Average D	0.830	0.466	0.561	2.284	1.896	0.389
SD	WWa	0.826	0.445	0.539	2.340	1.931	0.409
	WSa	0.832	0.457	0.549	2.288	1.902	0.386
	Average SD	0.829	0.451	0.544	2.314	1.916	0.397
D	WWb	0.825	0.449	0.544	2.286	1.885	0.401
	WSb	0.831	0.479	0.577	2.144	1.781	0.363
	Average D	0.828	0.464	0.560	2.215	1.833	0.382
SD	WWb	0.820	0.427	0.521	2.359	1.934	0.424
	WSb	0.830	0.453	0.546	2.264	1.879	0.385
	Average SD	0.825	0.440	0.533	2.311	1.906	0.405
D	WWc	0.830	0.443	0.533	2.209	1.834	0.375
	WSc	0.836	0.468	0.560	2.054	1.716	0.338
	Average D	0.833	0.456	0.547	2.131	1.775	0.356
SD	WWc	0.827	0.413	0.499	2.306	1.907	0.399
	WSc	0.836	0.444	0.531	2.175	1.817	0.358
	Average SD	0.831	0.428	0.515	2.240	1.862	0.378
WW: D vs. SD		bc	abc	abc	bc	bc	bc
WS: D vs. SD		ns	abc	abc	bc	bc	bc
Average D vs. average SD		b	abc	abc	bc	bc	bc

F-test at $P \leq 0.01$; the letters (a, b, c) mark stages of growth in which significance was detected between two average values; ns – not significant.

Table 8. The average values of photosynthetic parameters (TR$_0$/ABS, ET$_0$/ABS, ET$_0$/TR$_0$, ABS/RC, TR$_0$/RC, ET$_0$/RC, and DI$_0$/RC) of the groups of winter wheat genotypes (D and SD) in the well-watered (WW) and moderate short-term drought stress (WS) treatments and the average values of both treatments at the stages of the beginning of stem elongation (WWd, WSd), the developed flag leaf (WWe, WSe) and beginning of heading (WWf, WSf).

Group	Water treatment	Photosynthetic parameters					
		TR$_0$/ABS	ET$_0$/ABS	ET$_0$/TR$_0$	ABS/RC	TR$_0$/RC	DI$_0$/RC
D	WWd	0.835	0.505	0.604	2.004	0.332	0.332
	WSd	0.835	0.511	0.612	1.986	0.328	0.328
	Average D	0.835	0.508	0.608	1.995	0.330	0.330
SD	WWd	0.834	0.471	0.564	2.090	0.348	0.348
	WSd	0.833	0.479	0.574	2.078	0.347	0.347
	Average SD	0.834	0.475	0.569	2.084	0.348	0.348
D	WWe	0.833	0.547	0.657	2.219	0.371	0.371
	WSe	0.835	0.547	0.655	2.266	0.374	0.374
	Average D	0.834	0.547	0.656	2.243	0.373	0.373
SD	WWe	0.833	0.532	0.638	2.300	0.384	0.384
	WSe	0.832	0.529	0.635	2.337	0.395	0.395
	Average SD	0.833	0.530	0.637	2.319	0.390	0.390
D	WWf	0.832	0.542	0.651	2.221	0.374	0.374
	WSf	0.826	0.524	0.634	2.319	0.404	0.404
	Average D	0.829	0.533	0.642	2.270	0.389	0.389
SD	WWf	0.830	0.517	0.622	2.328	0.393	0.393
	WSf	0.818	0.500	0.611	2.456	0.450	0.450
	Average SD	0.824	0.508	0.617	2.392	0.421	0.421
WW: D vs. SD		ns	df	df	df	df	ns
WS: D vs. SD		f	df	df	df	df	ef
Average D vs. average SD		f	df	df	df	df	df

F-test at P≤0.01; the letters (d, e, f) mark stages of growth in which significance was detected between two average values; ns – not significant.

Table 9. The average values of photosynthetic parameters (ABS/CS_0, TR_0/CS_0, ET_0/CS_0, DI_0/CS_0, RC/CS_0, RC/ABS, $ET_0/(TR_0\text{-}ET_0)$) of the groups of winter wheat genotypes (D and SD) in the well-watered (WW) and moderate short-term drought stress (WS) treatments and the average values of both treatments at the tillering stages of growth (WWa, WSa, WWb, WSb, WWc, WSc).

Group	Water treatment	Photosynthetic parameters						
		ABS/CS_0	TR_0/CS_0	ET_0/CS_0	DI_0/CS_0	RC/CS_0	RC/ABS	$ET_0/(TR_0\text{-}ET_0)$
D	WWa	459.19	380.05	211.27	79.142	199.10	0.435	1.261
	WSa	450.76	375.14	212.16	75.617	200.22	0.445	1.314
	Average D	454.97	377.60	211.72	77.379	199.66	0.440	1.288
SD	WWa	458.79	378.75	203.96	80.043	196.46	0.429	1.177
	WSa	452.00	375.80	206.30	76.201	198.31	0.440	1.228
	Average SD	455.40	377.28	205.13	78.122	197.39	0.434	1.203
D	WWb	460.14	379.48	206.48	80.657	201.57	0.438	1.198
	WSb	447.61	371.84	214.49	75.766	209.11	0.468	1.374
	Average D	453.87	375.66	210.48	78.211	205.34	0.453	1.286
SD	WWb	458.46	375.87	195.95	82.589	194.74	0.425	1.096
	WSb	457.81	379.85	207.26	77.963	202.41	0.443	1.205
	Average SD	458.14	377.86	201.61	80.276	198.57	0.434	1.151
D	WWc	457.42	379.76	202.56	77.662	207.35	0.454	1.151
	WSc	449.21	375.33	210.26	73.881	219.14	0.488	1.281
	Average D	453.31	377.54	206.41	75.771	213.25	0.471	1.216
SD	WWc	465.25	384.67	192.08	80.582	202.12	0.435	1.002
	WSc	460.03	384.32	204.02	75.709	211.85	0.461	1.138
	Average SD	462.64	384.50	198.05	78.146	206.98	0.448	1.070
WW: D vs. SD		ns	ns	abc	c	bc	bc	abc
WS: D vs. SD		bc	bc	abc	ns	bc	bc	abc
Average D vs. SD		c	c	abc	bc	bc	bc	abc

F-test at $P \le 0.01$; the letters (a, b, c) mark stages of growth in which significance was detected between two average values; ns – not significant.

Table 10. The average values of photosynthetic parameters (ABS/CS$_0$, TR$_0$/CS$_0$, ET$_0$/CS$_0$, DI$_0$/CS$_0$, RC/CS$_0$, RC/ABS, ET$_0$/(TR$_0$-ET$_0$)) of the groups of winter wheat genotypes (D and SD) in the treatments well-watered (WW) and moderate short-term drought stress (WS) and the average values of both treatments at the stages of the beginning of stem elongation (WWd, WSd), the developed flag leaf (WWe, WSe), and beginning of heading (WWf, WSf).

Group	Water treatment	Photosynthetic parameters						
		ABS/CS$_0$	TR$_0$/CS$_0$	ET$_0$/CS$_0$	DI$_0$/CS$_0$	RC/CS$_0$	RC/ABS	ET$_0$/(TR$_0$-ET$_0$)
D	WWd	404.91	337.95	204.07	66.958	202.74	0.502	0.502
	WSd	411.51	343.50	209.70	68.009	207.66	0.506	0.506
	Average D	408.21	340.72	206.88	67.484	205.20	0.504	0.504
SD	WWd	414.33	345.33	195.01	69.002	198.80	0.481	0.481
	WSd	411.87	343.14	196.80	68.730	198.51	0.483	0.483
	Average SD	413.10	344.24	195.91	68.866	198.66	0.482	0.482
D	WWe	434.80	362.21	237.95	72.596	196.17	0.452	0.452
	WSe	435.45	363.56	237.90	71.894	192.44	0.443	0.443
	Average D	435.13	362.88	237.92	72.245	194.31	0.447	0.447
SD	WWe	447.37	372.78	237.75	74.590	195.02	0.436	0.436
	WSe	443.02	368.19	233.75	74.830	189.72	0.429	0.429
	Average SD	445.20	370.49	235.75	74.710	192.37	0.433	0.433
D	WWf	405.94	337.71	219.88	68.226	183.06	0.452	0.452
	WSf	413.52	341.40	216.01	72.128	178.35	0.433	0.433
	Average D	409.73	339.55	217.95	70.177	180.71	0.442	0.442
SD	WWf	398.41	329.77	205.15	68.640	174.03	0.434	0.434
	WSf	425.40	347.46	212.02	77.938	173.40	0.410	0.410
	Average SD	411.91	338.62	208.59	73.289	173.72	0.422	0.422
WW: D vs. SD		ef	ef	df	ns	f	df	df
WS: D vs. SD		ns	ns	d	f	df	df	df
Average D vs. SD		e	e	df	e	df	df	df

F-test at $P \leq 0.01$; the letters (d, e, f) mark stages of growth in which significance was detected between two average values; ns – not significant.

Table 11. Correlation coefficients for 15 photosynthetic parameters with their photosynthetic performance indices (PI_{ABS}) observed in the well-watered (WW) and the moderate short-term drought stress (WS) treatments at different stages of growth (a–f) of 14 winter wheat genotypes.

Para-meters	Correlation coefficients (r) for the photosynthetic parameters with their PI_{ABS} observed in WW and WS treatments at different growth stages (a–f) of 14 winter wheat genotypes											
	WW (well-watered)						WS (moderate short-term drought stress)					
	a	b	c	d	e	f	a	b	c	d	e	f
TR_0/ABS	0.82	0.58	0.58	0.60	0.58	0.54	0.67	0.38	0.31	0.60	0.84	0.89
ET_0/ABS	0.97	0.96	0.97	0.99	0.96	0.97	0.97	0.97	0.96	0.98	0.98	0.98
ET_0/TR_0	0.94	0.88	0.95	0.98	0.92	0.96	0.94	0.94	0.93	0.97	0.94	0.92
ABS/RC	-0.87	-0.89	-0.90	-0.89	-0.85	-0.49	-0.92	-0.95	-0.94	-0.89	-0.91	-0.92
TR_0/RC	-0.83	-0.80	-0.86	-0.86	-0.80	-0.34	-0.91	-0.94	-0.91	-0.88	-0.86	-0.91
ET_0/RC	0.28	0.33	0.54	0.51	-0.10	0.31	-0.19	-0.06	0.06	0.55	-0.17	-0.35
DI_0/RC	-0.90	-0.85	-0.88	-0.85	-0.88	-0.95	-0.90	-0.85	-0.87	-0.84	-0.94	-0.91
ABS/CS_0	-0.52	-0.03	-0.55	-0.67	-0.49	-0.12	-0.79	-0.63	-0.69	-0.56	-0.73	-0.81
TR_0/CS_0	-0.34	0.23	-0.48	-0.57	-0.37	-0.01	-0.72	-0.63	-0.71	-0.50	-0.59	-0.74
ET_0/CS_0	0.70	0.75	0.80	0.73	0.47	0.56	0.64	0.64	0.60	0.85	0.41	0.01
DI_0/CS_0	-0.81	-0.43	-0.62	-0.74	-0.72	-0.44	-0.81	-0.55	-0.53	-0.61	-0.89	-0.87
RC/CS_0	0.71	0.80	0.73	0.71	0.59	0.25	0.71	0.75	0.68	0.76	0.42	0.29
RC/ABS	0.88	0.89	0.90	0.89	0.84	0.58	0.91	0.94	0.95	0.89	0.91	0.94
TR_0/DI_0	0.81	0.59	0.58	0.62	0.59	0.29	0.65	0.40	0.34	0.63	0.84	0.90
$ET_0/(TR_0-ET_0)$	0.93	0.88	0.95	0.97	0.91	0.96	0.93	0.93	0.94	0.96	0.95	0.91

Significance of correlation coefficient: $r \geq 0.532$ *(*$P \leq 0.05$*);* $r \geq 0.661$ *(*$P \leq 0.01$*);* $r \geq 0.780$ *(*$P \leq 0.001$*).*

Besides that, in the WW treatment, correlation coefficients of the PI_{ABS} parameter between developmental stages were positive and significant ($P \leq 0.05$ and $P \leq 0.01$), or more precisely explained, a significant positive correlation was determined between the end of the tillering stage (a, b, c) with the results determined at the beginning of stem elongation stage (d) and the stage of the developed flag leaf (e). Furthermore, the same tendency of positive correlation coefficients was also estimated in the WS treatment (**Table 13**).

In the D group of genotypes, the highest grain yield was achieved by Kraljica, which has relatively higher values of PI_{ABS}, especially at stages e and f. In the SD group of genotypes, the genotypes Antonija, Apache, and Grandior have achieved higher grain yields, but their PI_{ABS} values were at the average of all 14 studied genotypes or even lower (**Tables 2**, **5**, and **6**), which is consistent with the research by *Gent and Kiyomoto (1997)*. They reported that genotypes with shorter plant height have better photosynthetic rates, but fewer leaf areas than genotypes with higher plant height.

The relatively higher PI_{ABS} values belonged to genotypes Alka and Srpanjka in both water treatments (WW and WS), especially at the tillering stage (a, b, and c), and Kraljica and Renata at the stages e and f, which belong to the D group of genotypes (**Tables 2**–**6**). Genotypes Antonija, Vulkan, and Žitarka, which belong to the SD group, had relatively lower values of PI_{ABS} (Tables 2-6). Similar results can be expected for other investigated photosynthetic parameters that are in strong positive correlations with PI_{ABS} and the opposite for photosynthetic parameters with strong negative correlations with PI_{ABS} (**Table 11**).

The majority of tested winter wheat genotypes had higher values of ET_0/ABS, ET_0/RC, $ET_0/(TR_0\text{-}ET_0)$, and PI_{ABS} in the WS treatment at the early stage of development (tillering stage; measurements a, b, c), when compared to WW treatment. This increase in photosynthetic electron transport at the early stage of development can be linked to the activation of mechanisms responsible for moderate short-term drought tolerance, which also agrees with earlier research (*Kovačević et al. 2013, 2017*), and this was particularly pronounced in winter wheat genotypes with a higher harvest index, grain yield per vegetation pot, and grain yield stability than the average of all tested winter wheat genotypes (*Kovačević et al. 2013*). *Kenny (2016)* also reported that drought-tolerant winter wheat genotype Plainsman showed a marked increase in photosynthetic performance index (PI) and the density of RC on chl *a* basis (RC/ABS) on the flag leaves after 14, 20, and 27 days of the drought stress, while drought sensitive winter wheat genotype Cappelle Desprez had the same responses after only 14 days of drought stress and the opposite responses after 27 days of the drought stress. Similar results were also published in the study by *Balouchi (2011)* that reported increasing F_v/F_m (TR_0/ABS) values of several Australian wheat genotypes after the drought stress treatment at the early stage of growth. Accordingly, *Loggini et al. (1999)* reported that a higher total rate of photosynthetic electron transport of drought-tolerant wheat genotypes in response to drought stress was probably sufficient to prevent the collection of excess energy in PSII. The absence of susceptibility to drought stress of frequently studied parameter TR_0/ABS or F_v/F_m was also observed in other cereal species because the photosynthetic apparatus is resistant to water deficiency (*Chaves et al. 2002, Cornic and Fresneau 2002, Kocheva et al. 2004, Hura et al. 2007*). Therefore, *Araus et al. (1998)* pointed out a significant ($P \leq 0.001$) positive correlation between TR_0/ABS (F_v/F_m) at the flag leaf stage and grain yield of 144 wheat genotypes in the rain-fed and 124 wheat genotypes in irrigation treatment, while *Špani*ć *et al. (2013)* did not determine it in the study of 10 winter wheat genotypes in approximately optimal conditions for the photosynthetic parameters TR_0/ABS or F_v/F_m and PI_{ABS}. *Öztürk and Aydin (2017)* reported significantly positive correlation coefficients of TR_0/ABS or F_v/F_m with grain yield of Turkish genotypes in arid field conditions. In our paper parameter, TR_0/ABS or F_v/F_m had the tendency of higher values in the D group of genotypes compared to the SD group, but genotypes of group D achieved lower grain yield than genotypes of the SD group (**Tables 2**, **7**–**8**).

Šlapakauskas and Ruzgas (2005) pointed out that the selection of winter wheat breeding material using the chl *a* fluorescence method is more precise when the genotypes or breeding lines are grouped according to the dates of maturity. The same authors pointed out those fluorescence parameters of the photosynthetic system II which correlated with plant characteristics and crop productivity. Therefore, the most yielding winter wheat genotype (Seda) had significantly shorter plant height than the control genotype (Širvinta) but the lowest electron transport rate.

Differences between genotypes in both water treatments, as well as the differences between groups of genotypes (D and SD), and differences between water treatments (WW and WS) were most evident in the photosynthetic parameter PI_{ABS}, as well as in parameters ET_0/ABS, ET_0/TR_0, and $ET_0/(TR_0\text{-}ET_0)$, which were strongly ($P \leq 0.01$) positively correlated with PI_{ABS}. The interaction between treatments and genotypes was significant ($P \leq 0.01$) alone in the ET_0/ABS and PI_{ABS} parameters at the flag leaf stage (**Tables 5**–**10**). This indicates that PI_{ABS} is a reliable parameter for distinguishing genotypes in different environmental conditions, as indicated by *Strasser et al. (2004)* and *Oukarroum et al. (2007)*.

In earlier studies on a similar set of winter wheat genotypes (*Kovačević et al. 2017*), it was estimated that genotypes of higher grain yields per vegetative pot had better water use efficiency, higher harvest index, and a better index of tolerance for moderate short-term drought stress, but increased values of the photosynthetic parameters ABS/CS_0, ET_0/CS_0 and DI_0/CS_0 had negative, while the higher value of PI_{ABS} had a positive influence on the mentioned agronomic values.

Table 12. The variances of error (MSe) for 16 photosynthetic parameters of 14 winter wheat genotypes observed from the tillering stage (a, b, c – Zadoks 26–29), the beginning of stem elongation (d – Zadoks 35–36), and the stage of flag leaf and beginning of heading (e, f – Zadoks 40–60).

Parameters	Growth stages						F-value between max and min MSe
	a	b	c	d	e	f	
TR_0/ABS	6.55E-05	7.17E-05	5.95E-05	7.4E-05	9.4E-05	0.000226	3.798 f/c
ET_0/ABS	0.000522	0.000402	0.000384	0.001685	0.000418	0.000729	4.388 d/c
ET_0/TR_0	0.000663	0.000542	0.000531	0.002243	0.000430	0.000758	4.224 d/c
ABS/RC	0.020394	0.009403	0.010678	0.020123	0.013578	0.024674	2.624 f/b
TR_0/RC	0.011791	0.005938	0.00698	0.011484	0.007454	0.017568	2.959 f/b
ET_0/RC	0.002195	0.002891	0.001702	0.005031	0.003204	0.006931	4.072 f/c
DI_0/RC	0.001537	0.00086	0.000744	0.001513	0.001432	0.002656	3.570 f/c
ABS/CS_0	642.2298	429.3138	399.5076	607.6204	514.3501	1093.48	2.737 f/c
TR_0/CS_0	375.7478	266.3739	251.0567	345.1635	314.1985	616.6178	2.456 f/c
ET_0/CS_0	168.7341	182.133	148.0446	310.5609	150.2557	343.4394	2.320 f/c
DI_0/CS_0	50.27009	37.93815	32.12376	49.74994	46.7558	105.1796	3.274 f/c
RC/CS_0	128.5756	93.36597	100.8596	108.4274	98.85192	213.7959	2.290 f/b
RC/ABS	0.000652	0.000356	0.00045	0.001045	0.000474	0.000664	2.936 d/b
TR_0/DI_0	0.070883	0.068417	0.068687	0.074955	0.102892	0.336772	4.922 f/b
$ET_0/(TR_0-ET_0)$	0.016988	0.013202	0.011782	0.078378	0.029047	0.036879	3.130 d/c
PI_{ABS}	0.202421	0.114868	0.123415	0.773382	0.378172	0.464701	6.733 d/b

Significance of F-test between maximum end minimum MSe: F ≥ 1.72 (P≤0.05); F ≥ 2.17 (P≤0.01); F ≥ 2.78 (P≤0.001).

Table 13. The correlation coefficients between PI_{ABS} of 14 winter wheat genotypes observed in different growth stages (a–f); above diagonal is WW treatment; below diagonal is WS treatment (WW – well-watered; WS – moderate short-term drought stress).

Growth stages	Correlations coefficients between PI_{ABS}					
	a	b	c	d	e	f
a		0.651	0.676	0.537	0.567	0.381
b	0.462		0.866	0.579	0.622	0.341
c	0.609	0.862		0.837	0.689	0.512
d	0.241	0.634	0.595		0.776	0.732
e	0.386	0.416	0.492	0.548		0.671
f	0.187	0.448	0.528	0.520	0.785	

Significance of correlation coefficient: r≥ 0.532 (P≤0.05); r≥ 0.661 (P≤0.01); r≥0.780 (P≤0.001).

Table 14. Correlation coefficient of PIABS with stem height observed in the pots trial, and PIABS and grain yield observed in field trials in two water application treatments (WW and WS) at different growth stages (a–f).

PIABS in water treatments and growth stages	Correlation coefficient between PIABS and stem heigh and grain yield					
	Stem height in field trial			Grain yield in field trials		
	2012	2013	Average	2012	2013	Average
PIABS in WWa	-0.294	-0.162	-0.222	-0.315	-0.184	-0.264
PIABS in WWb	-0.542	-0.494	-0.526	-0.311	-0.300	-0.338
PIABS in WWc	-0.720	-0.721	-0.738	-0.311	-0.436	-0.425
PIABS in WWd	-0.680	-0.637	-0.671	-0.182	-0.216	-0.224
PIABS in WWe	-0.602	-0.449	-0.524	-0.334	-0.257	-0.320
PIABS in WWf	-0.585	-0.480	-0.536	-0.219	-0.113	-0.174
PIABS in WSa	-0.448	-0.189	-0.303	-0.572	-0.148	-0.359
PIABS in WSb	-0.610	-0.612	-0.626	-0.305	-0.387	-0.391
PIABS in WSc	-0.604	-0.586	-0.608	-0.317	-0.393	-0.400
PIABS in WSd	-0.657	-0.741	-0.723	-0.119	-0.436	-0.337
PIABS in WSe	-0.342	-0.357	-0.360	-0.360	-0.373	-0.407
PIABS in WSf	-0.333	-0.417	-0.391	-0.043	-0.318	-0.225

Significance of correlation coefficient: r≥ 0.532 (P≤0.05); r≥ 0.661 (P≤0.01); r≥0.780 (P≤0.001).

Table 15. Correlation coefficient (r) between PIABS observed in pots trial in two water application treatments (WW and WS) at different growth stages (a–f), and 1000 grains mass observed in field trial, and spike number per vegetative pots.

PIABS in water treatments and growth stages	Correlation coefficient (r) between PIABS and 1000 grain mass and spike number					
	1000 grains mass observed in field trial			Spike number per vegetative pots		
	2012	2013	Average	WW	WS	Average
PIABS in WWa	0.000	-0.214	-0.121	0.153	-0.157	0.012
PIABS in WWb	-0.394	-0.548	-0.494	0.464	0.040	0.288
PIABS in WWc	-0.437	-0.657	-0.575	0.556	0.123	0.381
PIABS in WWd	-0.403	-0.528	-0.487	0.476	-0.010	0.270
PIABS in WWe	-0.333	-0.502	-0.439	0.292	-0.270	0.040
PIABS in WWf	-0.444	-0.455	-0.465	0.322	-0.153	0.113
PIABS in WSa	-0.006	-0.224	-0.129	0.074	-0.398	-0.147
PIABS in WSb	-0.516	-0.664	-0.617	0.589	0.256	0.461
PIABS in WSc	-0.551	-0.661	-0.631	0.531	0.089	0.348
PIABS in WSd	-0.303	-0.557	-0.457	0.351	-0.021	0.194
PIABS in WSe	0.012	-0.199	-0.107	-0.109	-0.405	-0.257
PIABS in WSf	-0.103	-0.303	-0.219	0.034	-0.187	-0.070

Significance of correlation coefficient: r≥ 0.532 (P≤0.05); r≥ 0.661 (P≤0.01); r≥0.780 (P≤0.001).

Table 16. Correlation coefficient (r) between PIABS and time of heading and leaf senescence observed in pots trial in two treatments of water application (WW and WS) at different growth stages (a-f).

PIABS in water treatments at different growth stage	Correlation coefficient (r) between PIABS and heading time and leaf senescence					
	Time of heading			Leaf senescence		
	WW	WS	Average	WW	WS	Average
PIABS in WWa	-0.170	-0.095	-0.169	-0.564	-0.191	-0.261
PIABS in WWb	-0.426	-0.349	-0.400	-0.457	-0.325	-0.387
PIABS in WWc	-0.473	-0.409	-0.446	-0.570	-0.533	-0.599
PIABS in WWd	-0.362	-0.308	-0.339	-0.474	-0.518	-0.556
PIABS in WWe	-0.423	-0.347	-0.420	-0.468	-0.511	-0.535
PIABS in WWf	-0.529	-0.479	-0.520	-0.103	-0.452	-0.415
PIABS in WSa	-0.153	-0.032	-0.148	-0.579	-0.032	-0.115
PIABS in WSb	-0.485	-0.417	-0.449	-0.305	-0.548	-0.564
PIABS in WSc	-0.509	-0.444	-0.492	-0.458	-0.619	-0.648
PIABS in WSd	-0.431	-0.405	-0.401	-0.516	-0.717	-0.759
PIABS in WSe	-0.189	-0.134	-0.150	-0.310	-0.523	-0.514
PIABS in WSf	-0.212	-0.209	-0.203	-0.458	-0.742	-0.749

Significance of correlation coefficient: r≥ 0.532 (P≤0.05); r≥ 0.661 (P≤0.01); r≥0.780 (P≤0.001).

Correlation coefficients between PIABS parameter of genotypes in WW and WS treatments have a negative sign for stem height (**Table 14**), 1000 grains mass (**Table 15**), time of heading, and leaf senescence (**Table 16**) confirming what was observed in the results shown in **Tables 2**, **3**, and **4**.

By using photosynthetic parameters, especially the photosynthetic performance index (PIABS), already at the earlier stages of development (from the beginning of tillering to the beginning of heading), it is possible to grow winter wheat genotypes with a specific habit in terms of stem height, early maturity, and tolerance to flag leaf senescence, as determined by *Gent and Kiyomoto (1997)*. It is noticeable that wheat genotypes with a lower stem have a lower grain yield (**Table 2**), which is indicated by mostly weak negative correlation coefficients between PIABS and grain yield in field experiments (**Table 14**), which may be influenced by special weather conditions and other environmental influences, particularly disease development at the later stages of development. Also, these dwarf genotypes (varieties) were created 30 to 40 years ago, which may be the cause of the negative correlations between PIABS parameter and grain yield predicted at early wheat growth stages because, during that time, significant progress was made in plant breeding.

The application of ChlF assessments (photosynthetic parameters) in the breeding programs has a valuable potential because of its interdependence with some important plant attributes such as stem height, time of heading, the mass of 1000 grains, and indirectly grain yield, while simultaneously it has fast, simple and non-invasive nature coupled with the possibility of usage at the stages of early growth and development. Similar methods can be applied to many species of cultivated plants with additional measurement of the leaf temperature in conditions of drought and high air temperatures, determining water use (WU) and water use efficiency (WUE) by different plant species (*Araus et al. 2008, Reynolds et al. 2009, Kovačević et al. 2015, Markulj Kulundžić et al. 2016, Josipović 2019*).

Case study: Winter Barley

Eight winter barley genotypes (Barun, Bingo, Zlatko, Vanessa, Rex, Tiffany, Lord, and Favorit) were tested in the multi-environmental field trials from 2004 to 2007 (4 years; four locations; two sowing densities; three replicates) and also in the vegetative pots experiment (including two more genotypes Titan and Bravo). In addition, every barley cultivar which was examined in the vegetative pots (ten genotypes) was tested in the multi-environmental field trials from 2009 to 2011 (3 years; five locations; two sowing densities, three replicates) (*Lalić et al. 2009, Lalić et al. 2012, Kovačević et al. 2015*). The area of the basic plots of winter barley genotypes in the multi-environmental field trials was the same as that of winter wheat genotypes (7.56 m^2).

Table 17. Correlation coefficients for photosynthetic performance index (PI_{ABS}), stability index of PI_{ABS} (SI of PI_{ABS}), water use (WU), and grain yield-based water use efficiency (WUE_G) of different winter barley cultivars, with biomass weight per pot, harvest index, and grain yield per pot in two treatments of water application (WW – well-watered; WS – moderate short-term drought stress), and treatment average.

Correlated variables	Biomass weight per pot			Harvest index			Grain yield per pot		
	WW	WS	Average	WW	WS	Average	WW	WS	Average
PI_{ABS} in WW	0.043	-0.153	-0.058	-0.155	-0.232	-0.198	-0.077	-0.327	-0.199
PI_{ABS} in WS	-0.004	-0.256	-0.134	0.123	0.186	0.159	0.123	-0.038	0.048
SI of PI_{ABS}	-0.077	-0.067	-0.073	0.411	0.584	0.511	0.284	0.465	0.379
WU in WW	0.647	0.731	0.705	0.361	0.185	0.287	0.803	0.789	0.817
WU in WS	0.530	0.686	0.622	0.212	-0.017	0.107	0.574	0.559	0.581
WUE_G in WW	0.536	0.621	0.592	0.657	0.361	0.535	0.992	0.881	0.964
WUE_G in WS	0.317	0.460	0.398	0.708	0.617	0.688	0.867	0.978	0.943

$r \geq 0.632$ $P \leq 0.05$; $r \geq 0.765$ $P \leq 0.01$; $r \geq 0.872$ $P \leq 0.001$.

PI_{ABS} in WW and PI_{ABS} in WS = photosynthetic performance index (PI_{ABS}) for every of ten barley genotypes in WW and WS water treatments in pot experiments. WU in WW and WU in WS = the total water use from sowing to ripening. WUE_G in WW and WUE_G in WS = grain yield per pot/water use per pot (GYP/WU) for every of ten barley genotypes in WW and WS water treatments in pots experiments. SI of PI_{ABS} (stability index of PI_{ABS}) = PI_{ABS} in WS/ PI_{ABS} in WW for every of ten barley genotypes.

Note: Table 17 is partially taken from the work of Kovačević et al. (2015).

Grain yield and ecovalence (W_i) (*Wricke 1962*) measured in the multi-environmental field trials were used in correlation analyses along with variables from the pot trial (PI_{ABS}, biomass weight per pot – BWP, harvest index – HI, grain yield-based water use efficiency of different winter barley cultivars – WUE_G, and grain yield per pot – GYP). There are strong positive correlations between water use (WU), WUE_G, and GYP, measured in vegetative pots (**Table 17**), with grain yield measured in multi-environment field trials (**Table 18**). Also, strong correlations in the negative direction of the same indicators measured in vegetative pots indicate the high stability of high-yielding genotypes in the winter barley multi-environmental trials (**Table 18**) (*Kovačević et al. 2015*).

Table 18. Correlation coefficients of PI_{ABS}, stability index of PI_{ABS} (SI of PI_{ABS}), WU, WUE_G, and grain yield per pot (GYP) of winter barley genotypes which were tested in the pot trial (WW – well-watered; WS – moderate short-term drought stress) with grain yield and ecovalence for grain yield (Wi) of the same winter barley genotypes from multi-environmental field trials in two sowing densities (350 and 450 seeds m^{-2}).

Correlation coefficients between experimental data in vegetation pots and grain yield in multi-environment field experiments								
Parameters on the basis of pot trial	Trial with eight cultivars				Trial with ten cultivars			
	Sowing density, (seeds m^{-2})		Average	W_i	Sowing density, (seeds m^{-2})		Average	W_i
	300	450			300	450		
PI_{ABS} in WW	-0.458	-0.458	-0.459	0.220	-0.433	-0.675	-0.568	0.283
PI_{ABS} in WS	-0.205	-0.205	-0.207	0.257	-0.425	-0.541	-0.494	0.328
SI of PI_{ABS}	0.454	0.450	0.451	0.049	0.155	0.366	0.269	0.050
WU in WW	0.922	0.926	0.925	-0.823	0.720	0.773	0.761	-0.470
WU in WS	0.775	0.792	0.786	-0.616	0.791	0.828	0.825	-0.623
WUE_G in WW	0.776	0.791	0.782	-0.812	0.506	0.447	0.486	-0.578
WUE_G in WS	0.808	0.839	0.823	-0.854	0.307	0.370	0.348	-0.441
GYP in WW	0.812	0.827	0.819	-0.827	0.568	0.529	0.559	-0.587
GYP in WS	0.865	0.896	0.881	-0.871	0.451	0.519	0.497	-0.578

$r \geq 0.707$ $P \leq 0.05$; $r \geq 0.834$ $P \leq 0.01$; $r \geq 0.925$ $P \leq 0.001$ for trial with eight cultivars.

$r \geq 0.632$ $P \leq 0.05$; $r \geq 0.765$ $P \leq 0.01$; $r \geq 0.872$ $P \leq 0.001$ for trial with ten cultivars.

PI_{ABS} in WW and PI_{ABS} in WS = photosynthetic performance index (PI_{ABS}) for every of ten barley genotypes in WW and WS water treatments in pot experiments. WU in WW and WU in WS = the total water use from sowing to ripening. WUE_G in WW and WUE_G in WS = grain yield per pot/water use per pot (GYP/WU) for every of ten barley genotypes in WW and WS water treatments in pots experiments. SI of PI_{ABS} (stability index of PI_{ABS}) = PI_{ABS} in WS/ PI_{ABS} in WW for every of ten barley genotypes.

Note: Table 18 is partially taken from the work of Kovačević et al. (2015).

Conclusion

Rational water use of plant cultivars could be a good indicator for plant production in water-limited conditions (*Reynolds et al. 2007, Araus et al. 2008, Blum 2009, Yong'an et al. 2010*). Several authors have studied the relationship between water-use efficiency (WUE) and various agronomic traits and pointed out high positive correlation coefficients for WUE with grain yield and harvest index (*Yong'an et al. 2010, Shamsi et al. 2010, Kovačević et al. 2015*).

In experiments with ten genotypes of winter wheat in vegetation pots, it was determined that lower values of ABS/CS_0, ET_0/CS_0, and DI_0/CS_0, and higher values of PI_{ABS}, measured on winter wheat genotypes at the tillering stage of growth in drought stress conditions could indicate higher tolerance on drought stress conditions (*Kovačević et al. 2013*). Higher values of ABS/CS_0 and ET_0/CS_0 in some genotypes cause an increased dissipation (DI_0/CS_0), which has a negative impact on grain yield per vegetative pot, water use efficiency of genotypes, and stability of grain yield (*Kovačević et al. 2017*). This is also confirmed by the strong negative correlations between the parameters of photosynthesis and agronomic traits. Results of the studied photosynthetic efficiency parameters of wheat cultivars were also a good predictor for important agronomic traits, especially when they were detected at the early stage of growth (*Kovačević et al. 2017*). According to the values of grain yield per pot, water use efficiency, harvest index, stability of grain yield, and some photosynthetic parameters (ABS/CS_0, ET_0/CS_0, DI_0/CS_0), it was detected that genotypes Katarina, Lucija, and Alka were singled out as the genetic material with a favourable set of traits which could be used in further breeding for drought stress tolerance (*Kovačević et al. 2017*).

In the aforementioned experiments with winter wheat and winter barley, the photosynthetic parameter PI_{ABS} measured in vegetative pots did not show stronger correlations with grain yield, only weaker negative correlations (**Tables 14**, **17**, and **18**), but in the experiments with winter barley, weaker positive correlations were estimated between stability index (SI) of PI_{ABS} and grain yield in multi-environmental field trials and harvest index (**Tables 17** and **18**).

In the winter wheat and winter barley breeding, with the intention to select specific habitus of genotypes, it is possible to obtain useful data through experiments in vegetation pots and the application of parameters by measuring the fluorescence of chlorophyll *a* (JIP test), which, along with other indicators, can be an auxiliary tool in wheat and barley breeding before sowing in field trials.

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Part 5

Chlorophyll *a* Fluorescence and Beyond: Recent Advancements and Future Prospects

Chlorophyll *a* Fluorescence and Beyond: Recent Advancements and Future Prospects

Domagoj Šimić and Selma Mlinarić

Chlorophyll fluorescence (ChlF) has been used for more than two decades to study photosynthesis in plants, algae, and bacteria as a non-invasive measurement of photosystem II activity (*Maxwell and Johnson 2000*). *Lepeduš (2023)* gave a historical overview of the ChlF methods utilized in Croatia over the last twenty years measured in many plant species including non-crop plants such as sour cherries, sweet cherries, apple, plum (*Mihaljević and Viljevac Vuletić 2023*), common fig (*Mlinarić et al. 2023*), cabbage, basil, and silver knapweed (*Antunović Dunić and Peršić 2023*).

The ChlF techniques are non-invasive, very sensitive, and fast, which was shown to be a powerful tool for providing a reliable source of information on plant condition. The estimation of the photosynthetic performance and the assessment of photosynthetic electron transport and related photosynthetic processes give a good insight ability of a plant to tolerate various environmental stresses. It was shown that ChlF has been useful for evaluating stress and adverse effects, but also for detecting the response of plants to weak environmental signals, potential pollutants such as heavy metals and metalloids, silver nanoparticles and industrial by-products, as well as to moderate mechanical force (*Vidaković-Cifrek and Tkalec 2023, Žuna Pfeiffer et al. 2023*). It has been proven a useful method to study adaptation of various species to different light conditions (*Mlinarić et al. 2023, Vidaković-Cifrek and Tkalec 2023*), response to heat (*Mlinarić et al. 2023, Vidaković-Cifrek and Tkalec 2023*) or chilling (*Mlinarić et al. 2023,*), drought (*Antunović Dunić and Peršić 2023, Vidaković-Cifrek and Tkalec 2023*), and salinity (*Antunović Dunić and Peršić 2023*).

According to the *Web of Science Core Collection (2019)* database search using the keyword "chlorophyll fluorescence", there are thousands of articles dealing with the method. Over the last twenty years, the total number of articles published in indexed journals was steadily increasing from more than 500 per year in 2001 to more than 2000 articles/year in 2021 (**Figure 1**). Markedly, there was a slight decrease in 2022, indicating possibly reducing interest in the subject.

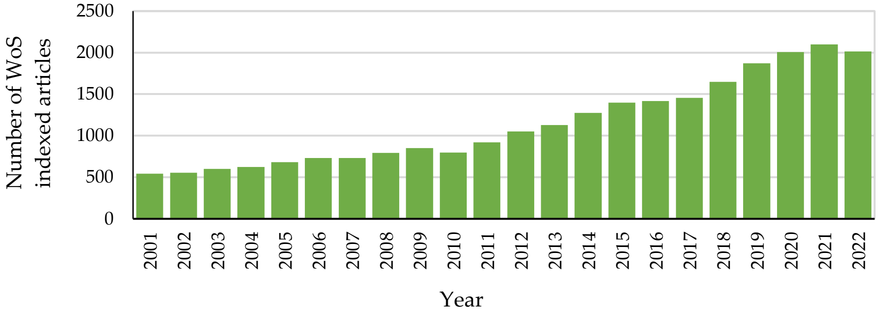

Figure 1. The total number of articles published in indexed journals from 2001 to 2022, retrieved from the Web of Science Core Collection database using the keyword "chlorophyll fluorescence" (source: *Web of Science Core Collection 2019*).

Nevertheless, ChlF is still widely used in plant research, and new developments in technology and analysis have improved its usefulness. A few recent advancements include:

1. Imaging of chlorophyll fluorescence: Fluorescence imaging techniques can provide spatially resolved information about photosynthesis in leaves, allowing researchers to study the heterogeneity of photosynthetic activity within leaves (*Lenk et al. 2007, Gorbe and Calatayud 2012, Ivanov and Bernards 2016*, *Lazarević 2023*).

2. Multispectral chlorophyll fluorescence and imaging reflectance: By measuring fluorescence emission at different wavelengths, researchers can distinguish between different photosynthetic pigments and study their contributions to photosynthesis (*Lenk et al. 2007*). Different types of stress induce diverse fluorescence and spectral characteristics that could be used to evaluate the physiological status of plants. Therefore, newly designed high-throughput crop physiology monitoring systems and the corresponding monitoring methods are based on chlorophyll fluorescence and multispectral imaging, which can estimate the degree of stress as well as provide the basis for crop management and provide the possibility of automatic machine diagnosis (*Wang et al. 2018, Javornik et al. 2023*).

3. Proximal and remote sensing of chlorophyll fluorescence: Novel communication devices (Internet of Things - IoT), emerging phenotyping technologies

and methods (high-throughput phenotyping), and data science techniques (machine learning) show promise in the integration of ChlF proximal sensing into larger frameworks (*Šimić et al. 2022*). Satellite-based instruments can measure chlorophyll fluorescence from entire ecosystems, providing information about the productivity of vegetation on a global scale (*Peng et al. 2020*).

4. Applications in plant breeding: ChlF can be used as a non-destructive, high-throughput screening tool for identifying plants with improved photosynthetic efficiency, which could lead to more productive crop varieties, e.g. in wheat and barley (*Kovačević et al. 2023, Španić et al. 2023),* maize *(Mazur et al. 2023)*, soybean (*Matoša Kočar et al. 2023*), and sunflower (*Markulj Kulundžić et al. 2023*).

5. Combination with other techniques: ChlF and/or ChlF imaging in combination with other measurement techniques and instrumentation can provide a unique research tool that will help us answer novel questions. Various techniques, such as infra-red gas exchange (IRGA), could correlate PSII photosynthetic efficiency directly to the CO_2 assimilation rate (*Buffon et al. 2018, Calzadilla et al. 2022*). Hyperspectral imaging, such as the Normalized Difference Vegetation Index (NDVI), could be used to detect various diseases and stresses by distinguishing chlorophyll degradation based on photosynthetic efficiency and spectral signatures (*Bednaříková et al. 2020, Begović et al. 2020*). Combination with other imaging technologies is also proving an extremely powerful tool in the development of large-scale phenotyping protocols and platforms (*Murchie and Lawson 2013*).

In the future, ChlF is expected to play an increasingly important role in agriculture, forestry, and climate change research. Some potential future prospects comprise:

1. Precision agriculture: Chlorophyll fluorescence can be used to optimize crop yield by detecting early signs of stress or disease and adjusting irrigation and fertilizer applications accordingly (*Chang et al. 2020, Sishodia et al. 2020*).

2. Urban farming: ChlF can be used to screen crops growing under artificial lighting, especially LED light technology in greenhouses and vertical farming all year-round, without the threat of extreme weather events, to supply growing food demands (*Darko et al. 2014, Sytar et al. 2021, Janeeshma et al. 2022*).

3. Forestry management: Chlorophyll fluorescence can be used to monitor the health of forests and identify areas at risk of pest infestation or disease outbreaks. Informative potential of the method could give a broader understanding of the functional traits and physiological responses of pure and mixed forests to environmental factors (*Pollastrini et al. 2017, Bantis et al. 2020, Pollastrini et al. 2022, Swoczyna et al. 2022*).

4. Aquatic photosynthesis: ChlF can be measured at different scales or cellular organization levels, from coral reef surfaces to the isolated chloroplasts in suspensions. Fluorometers used in aquatic environments provide information on

temporal changes in photosynthetic performance under ambient conditions and/or extreme environments that can be correlated with primary productivity. However, a combination with other techniques is necessary to understand the physiological responses for variations in aquatic productivity (*Bhagooli et al. 2021, Muñoz-Fernández et al. 2021, Wang et al. 2021*).

5. Climate change research: Chlorophyll fluorescence can be used to monitor the response of plants to changing environmental conditions, such as increased CO_2 levels or temperature, and to better understand the impact of climate change on global ecosystems. The method could serve for identification of the healthier or less suitable species for future environmental ecology and management (*Kalaji et al. 2011, Rasineni and Reddy 2013, Bantis et al. 2020, Pollastrini et al. 2020*).

6. Bioenergy: Chlorophyll fluorescence can be used to screen for photosynthetic efficiency in algae and other microorganisms, which can then be used to produce biofuels (*Zhou et al. 2013, Gomes et al. 2017, Vitale et al. 2023*).

Overall, chlorophyll fluorescence remains an important tool for understanding photosynthesis and its response to environmental stress, the health of plants and ecosystems. Continued advancements in technology and analysis will further improve its usefulness. Its future prospects are bright, with many potential applications in agriculture, urban farming, forestry, aquatic research, climate change, and bioenergy research.

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