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Detection of multi-nutrients deficiency in cereal plants by the use of chlorophyll fluorescence

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Abstract: Nutrient deficiency (ND) stands as a prominent environmental factor that significantly impacts global plant growth and productivity. While numerous methods have been employed for detecting nutrient deficiencies in plants, many of them are invasive, time-consuming, and costly. In contrast, chlorophyll fluorescence (ChlF) signals have emerged as a non-destructive tool for the identification of specific nutrient deficiencies, such as nitrogen (N), phosphorus (P), and potassium (K), across various plant species. In this pioneering study, ChlF measurements were employed for the first time to detect a combination of nutrient deficiencies, including deficiencies in nitrogen and phosphorus (–NP), nitrogen and potassium (–NK), potassium and phosphorus (–KP), and a complete NPK deficiency (–NPK). The experiment was conducted using wheat (*Triticum aestivum*) and maize (*Zea mays*) plants, which were grown under controlled laboratory conditions. An optimal hydroponic system was established to facilitate eight experimental conditions, namely: control, –N, –P, –K, –NP, –NK, –KP, and –NPK. Measurements were systematically collected at two-day intervals over a span of 24 days. Our findings demonstrate that chlorophyll fluorescence signals can enable the differentiation of various nutrient deficiencies even prior to the onset of observable symptoms. Furthermore, the examination of chlorophyll fluorescence parameters enables us not only to identify a singular macronutrient deficiency but also to detect multiple macronutrient deficiencies concurrently in a plant.

Keywords: abiotic stress, JIP test, photosynthesis, *Triticum aestivum*, *Zea mays*

INTRODUCTION

Nutrients are essential for proper plant growth and physiological functions efficiently work. Soil minerals and organic materials, as well as organic or inorganic fertilisers, are applied to absorb the essential components. To utilise these nutrients efficiently, light, heat, and water must be adequately supplied. Wealth of crop yields depends also on regional practices and prevention strategies against biotic and abiotic stress factors. Optimum nutrient range as well as a minimum requirement level changes due to plant species. Nutrient deficiency symptoms occur after exposure below the minuscule amount of species needs. Excessive nutrient access negatively affects plant development owing toxicity. Consequently, the correct application rate and distribution of macro components is vital (Silva and Uchida, 2000). Several experts contend that categorising nutrients as macronutrients or micronutrients is difficult to justify biologically. Mengel and Kirkby (1987) advocated classifying important elements based on their biochemical activity and physiological function. Plant nutrients are classified into four fundamental classes in this categorisation. Nitrogen, phosphorus and potassium belong successively to the first three basic groups.

Nitrogen is a mineral nutrient classified to a first group that are part of carbon compounds. Amino acids, amides, proteins, nucleic acids, nucleotides, coenzymes, and hexoamines all include N (Evans and Sorger, 1966; Mengel and Kirkby, 1987). This element may be absorbed by plants in the form of nitrate (NO_3) and ammonium (NH_4^+) ions. Carbon (C), hydrogen (H), oxygen (O) and sulphur (S) amalgamate biologically with N to form amino acids, which are responsible for the building blocks of proteins. These molecules enable the creation of protoplasm, the location of cellular division and directly influence plant

increase and formation. Nitrogen is essential for all enzymatic processes since all plant enzymes are made from proteins. Because N is a significant component of the chlorophyll molecule, it is required for photosynthesis. This element is involved in the formation of various vitamins. N increases the quality and quantity of dry matter in leafy vegetables as well as the protein content of cereal crops.

Phosphorus is a mineral nutrient that belongs to the second group, which is distinguished by energy storage or structural integrity. This element is found in sugar phosphates, nucleic acids, nucleotides, coenzymes, phospholipids, phytic acid, and other compounds. It has vital functions in ATP reactions (Evans and Sorger, 1966; Mengel and Kirkby, 1987). Plants can obtain phosphorus as orthophosphate ions (HPO_4^{2-}, $H_2PO_4^-$). Phosphorus is essential for energy cache and transmission in photosynthesis and respiration as ADP and ATP (adenosine di- and triphosphate) and DPN and TPN (di- and triphosphopyridine nucleotide). Phosphorus is a key element of RNA and DNA structures, both of which are main ingredients of genetic information. The broadest proportion of P resides in well-developed plant seeds. In new cells, like shoots and root tips, where metabolism is vigorous and cell division is immediate, P is demanded in enormous amounts. This particular macronutrient is indispensable for root growth, flower formation, seed germination and fruit set. It has been found that phosphorus not only enhances crop quality but also assists in decreasing disease incidence in studied plants.

Potassium was designated in the third group of mineral nutrients which remains in ionic form. More than 40 enzymes require K as a cofactor. The primary cation in the generation and maintenance of cell turgor and electroneutrality (Evans and Sorger, 1966; Mengel and Kirkby, 1987). This element can be taken up by plants in the form of ion K^+. In contrast to N and P, K does not generate any essential organic molecules in the plant that include carbon atoms covalently attached to hydrogen atoms. In spite of that potassium is obligatory for development because it is an enzyme activator that accelerates metabolism. Potassium regulates the stomata aperture by avoiding superfluous transpiration, which maintains high turgor pressure and keeps the plant cool. In photosynthesis K is responsible for regulating the equilibrium of electrical charges at the site of ATP generation. In plant development, K promotes translocation of photosythates (sugars) and distribution, particularly to fruits and roots. It contributes to protein synthesis by assisting in the generation of ATP. Potassium has been found to promote plant disease resistance, grain and seed size, and the quality of fruits and vegetables (Silva and Uchida, 2000).

Nutrient deficiencies can also lead to changes in the structure of the plant's leaves, such as yellowing, stunted growth, and reduced chlorophyll content (Veazie *et al.*, 2020). However, usually these visual changes happen with a delay (take a few weeks) (Jose *et al.*, 2021), while by measuring chlorophyll fluorescence of the leaves, we can detect these changes and diagnose nutrient deficiency after a few days, allowing us to take corrective action. Thus, the ability to detect nutrient deficiencies early and respond quickly (Samborska *et al.*, 2019) is essential for ensuring the success of agricultural and horticultural endeavours.

The most crucial biological process on Earth is photosynthesis, which enable the existence of life and maintains the equilibrium of the biosphere. As a result of this multi-step and exceedingly intricate process, the energy of absorbed photons is transformed into stable chemical energy of organic compounds. The following simplified formula can be used to describe photosynthesis in general: $CO_2 + H_2O$ + luminous energy $\rightarrow CH_2O + O_2$. Solar energy (light) reaching the leaf surface in the form of a photon flux is absorbed by photosynthetic dye molecules, mainly chlorophyll, in light-harvesting complexes. From these complexes (also known as antenna pigment molecules), the absorbed energy can be transferred to the reaction centers of PSII and PSI in the form of electron excitation of the dye molecules and used to initiate photochemical reactions. Before reaching the reaction center, part of the excitation energy is squandered as warmth or reemitted as chlorophyll fluorescence (Kalaji and Łoboda, 2010).

Chlorophyll *a* fluorescence measurements are used to monitor the physiological condition under environmental stresses of all photosynthetic organisms (Živčak *et al.*, 2014; Goltsev *et al.*, 2016; Kalaji *et al.,* 2016; Kalaji *et al.,* 2018b; Samborska *et al.*, 2018; Samborska *et al.*, 2019; Loudari *et al.*, 2020; Samborska-Skutnik *et al.*, 2020; Vukelić *et al.*, 2021). There are many examples of successful application of ChlF for studying the reaction of photosynthetic apparatus to high and low temperature (Alam, Nair and Jacob, 2005; Oukarroum, Goltsev and Strasser, 2013; Kalaji *et al.*, 2018a), drought (Goltsev *et al.*, 2012; Dąbrowski *et al.*, 2019), salinity (Kan *et al.*, 2017), heavy metals (Azevedo, Glória Pinto and Santos, 2005; Bayçu *et al.*, 2017). Moreover, ChlF is used in ecological (Bąba *et al.*, 2016), climate change (Drusch *et al.*, 2017; Balazy *et al.*, 2018; Pflug *et al.*, 2018), and vegetation research (Li *et al.*, 2018). The measurements are simple, non-invasive and fast (depending on the methodology, they take between a few seconds to a few minutes) (Tol van der, Verhoef and Rosema, 2009). The technique is a highly sensitive method for identifying changes in the overall bioenergetic status of a plants (Kalaji and Łoboda, 2010).

Researchers have been working to understand how changes of photosynthesis process provides valuable information for improving the growth and health of plants and sustainability (Pontes, Rodriguez and Santiago, 2019). Photosynthesis and chlorophyll fluorescence (ChlF) are closely related processes that serve to detect the impact of various stress factors on plants (Kalaji *et al.*, 2016) and the correlation between photosynthesis, chlorophyll fluorescence and detection of nutrient deficiency in plants is a crucial area of research (Ripoll *et al.*, 2016).

Chlorophyll fluorescence signals have been proved to be used to assess the physiological status of plants and to detect nutrients deficiency in a fast and nondestructive way (Frydenvang *et al.*, 2015). When plants lack a certain essential nutrient, such as N, P, or K (Kalaji *et al.*, 2014b), the efficiency of photosynthesis decreases. As a result, the chlorophyll fluorescence emitted by the plant increases.

Up-to-date most of conducted researches have concentrated on analysing the effect of single nutrient deficiency (Aleksandrov *et al.*, 2014; Bosa *et al.*, 2014; Brestic *et al.*, 2014; Kalaji *et al.*, 2014a; Kalaji *et al.*, 2014b; Kalaji *et al.*, 2014c; Kalaji *et al.*, 2016; Cetner *et al.*, 2017; Kalaji *et al.*, 2017a; Kalaji *et al.*, 2017b; Kalaji *et al.*, 2018b; Kalaji *et al.*, 2018c; Bąba *et al.*, 2019; Cetner *et al.*, 2020; Kusaka *et al.*, 2021; Esmaeili *et al.*, 2022; Sieczko *et al.*, 2022; Lotfi *et al.*, 2022; Jaszczuk *et al.*, 2023) or triple deficiency such as NPK (Horaczek *et al.*, 2020). Based on the guidance of Prof. Hazem M. Kalaji, a distinguished expert in this field with over

15 years of dedicated research (personal communication), the objective of this study was to investigate the impact of multi-nutrient deficiencies (NP, NK, KP, and NPK) on the photosynthetic efficiency of maize and wheat plants. We tested H_0 hypothesis of no differences in leaf nutrient status as detected by chlorophyll fluorescence among control (no-deficiency), single- and multi-deficient maize and wheat plants. To our knowledge, this work is first of its kind, worldwide.

MATERIALS AND METHODS

PLANTS GROWTH CONDITIONS AND EXPERIMENT DESIGN

The experiment was conducted at the photosynthesis laboratory – Warsaw University of Life Sciences (SGGW) between 26th October and 19th of November 2022. The winter wheat cultivars 'APOSTEL' from IGP Polska sp. z o.o. sp. k. Company and maize 'LG 30.273' from Limagrain Polska sp. z o.o. company were used for the experiment.

Under lab conditions, plants were illuminated by LED panel (Biogenet Company, Józefów, Poland) between 8:00 a.m. to 8:00 p.m. (Φ_e ~139.9 W·m^{-2}; PPFD ~721.4 μmol·m^{-2}·s^{-1}, lx ~15.767 lm·m^{-2}). The microclimate parameters in the laboratory were as the following: temperature ~22°C, CO_2 level ~420 ppm, and relative humidity (*RH*) ~50%.

Seedlings grown on full nutrient Hoagland solution of pH ca. 5.1 (Hoagland and Arnon 1950) prior to stress application. The nutrient deficiency treatment was based on prepared solutions that lack one or more of the following nutrients: N, P, K, NP, NK, KP, and NPK. Nutrient deficiency stress was applied at 56 days after sowing (DAS) and plants grown as a control remained in Hoagland's with full nutrients solution (Tabs. 1, 2).

Three plants were selected from each experimental variant for measurement. The study was conducted on the second, third and fourth fully developed leaf of each plant taking three measurements on each leaf, resulting in 27 measurements from one environment. Samples from 48 plants (24 maize and 24 wheat) were taken from the top, middle and bottom of the leaf blade (Kalaji *et al.*, 2018b).

CHLOROPHYLL FLUORESCENCE MEASUREMENTS

The chlorophyll fluorescence parameters (JIP-test) were measured at 57, 59, 61, 63, 65, 67, 69, 72, 73 and 75 DAS (day after sowing) using a HandyPEA fluorimeter (Hansatech Instruments Company, King's Lynn, Norfolk, UK) after 30 min of dark adaptation, however measurements were done under low green light that does not initiate notable photosynthesis (Roldán *et al.*, 2006). The following measurement protocol was applied: measurement time – 1.0 s, actinic light intensity – 3500 μmol·m^{-2}·s^{-1}, wavelength – 635 ±10 nm. One measurement resulted in the detection of 118 points within 1 s. The JIP test described by Strasser, Srivastava and Tsimilli-Michael (2000) was used calculate the characteristic points of the photoinduced chlorophyll fluorescence transients to specific parameters of the light phase of photosynthesis. From 62 measured and calculated ChlF parameters, based on our previous experiments and literature (Kalaji *et al.* 2017b), we selected ChlF parameters most responsive to single and multiple nutrient deficiency. The list is presented in Table 3.

Table 1. Concentrations of chemical compounds in modified Hoagland solution in control and deficiency solutions[1)]

1.0 molar stock solution	Nutrient concentration in solution (cm^3·dm^{-3})							
	control	deficiency solution						
		–K	–N	–P	–NPK	–NP	–NK	–KP
$Ca(NO_3)_2 \cdot 4H_2O$	4	4	–	4	–	–	–	4
KNO_3	6	–	–	6	–	–	–	–
$MgSO_4 \cdot 7H_2O$	2	2	2	2	2	2	2	2
$NH_4H_2PO_4$	2	2	–	–	–	–	–	–
$NaNO_3$	–	6	–	–	–	–	–	–
$CaCl_2$ anhydrous	–	–	4	–	4	4	4	–
KCl	–	–	2	–	–	–	–	–
NaH_2PO_4	–	–	2	–	–	–	2	–
NH_4NO_3	–	–	–	1	–	–	–	4
1% iron citrate	2	2	2	2	2	2	2	2
Micronutrients (solution A)	1	1	–	1	1	1	1	1
Micronutrients (solution B)	–	–	1	–	–	–	–	–
K_2SO_4	–	–	–	–	–	3	–	–

[1)] The composition of the various culture media of minerals was achieved by addition *X* cm^3 of concentrated stock solution (1 mol·dm^{-3}) of corresponding component per 1 dm^3 of medium.
Source: own elaboration.

Table 2. Salts containing micronutrients used in modified Hoagland solution

Salts containing micronutrient	Quantity (g dm^{-3} H_2O) in	
	solution A	solution B
H_3BO_3	2.85	2.85
$MnSO_4 \cdot 4H_2O$	1.10	–
$ZnSO_4 \cdot 7H_2O$	0.28	–
$CuSO_4 \cdot 5H_2O$	0.10	–
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.02	–
NaCl	3.12	3.12
$MnCl_2 \cdot 4H_2O$	–	0.93
$ZnCl_2$	–	0.13
$CuCl_2 \cdot 2H_2O$	–	0.07
MoO_2	–	0.02

Source: Aleksandrov (2019), p. 5.

Table 3. Summary of selected measured and calculated chlorophyll *a* fluorescence parameters used in this work

Parameter	Definition
F_V/F_M	Maximum quantum yield of primary photochemical reactions at time zero (at $t = 0$), which proves the probability that the energy of absorbed photons (excitons migrating along an antenna) can be trapped by PSII RCs.
PI_{total}	Total performance index, indicating the integral functional activity of PSII, PSI, and intersystem electron transport chain.
ABS/CS_o	Energy absorption per unit cross section (CS) of a photosynthesising object and chlorophyll (at $t = 0$). This quantity represents the amount of photon energy absorbed by the antenna associated with active and inactive reaction centers of PSII.
TR_o/CS_o	Energy flux trapped by PSII reaction centers per unit cross section (CS) of a photosynthesising object at $t = 0$.
DI_o/CS_o	Dissipation of energy in PSII per unit cross section (CS) of a photosynthesising object at $t = 0$.
ET_o/CS_o	Electron flux through PSII per unit cross section (CS) of a photosynthesising object at $t = 0$.

Source: own elaboration based on: Strasser, Tsimilli-Michael and Srivastava (2004), Guidi and Degl'Innocenti (2011), Stirbet and Govindjee (2011), Brestic *et al.* (2014), Kalaji *et al.* (2014b), and Kalaji *et al.* (2017b).

STATISTICAL ANALYSIS

Principal component analysis was used to summarise the relationships among six selected chlorophyll fluorescence parameters, measured during ten time steps (t_0–t_9) in maize under single and multiple nutrient deficiency. All the analyses were performed in the R v.4.0.3 (R Core Team, 2020). One-way ANOVA with subsequent Tukey's post-hoc were used for pairwise comparisons of average values of selected chlorophyll *a* fluorescence parameters between control and single- and multiple nutrient deficiency treatments. The analyses were performed for all time points (t_0–t_9) separately. Prior to the analyses the normality of the variables were examined with the Shapiro–Wilk test and transformed where needed. The hypothesis of lack of differences among means was tested at significance level $\alpha = 0.01$. The analyses were conducted in Statistica v13.1. (TIBCO Software Inc., 2017).

RESULTS

VISIBLE SYMPTOMS

Significant visual symptoms of nutrient deficiency were observed in stressed plants compared to the control group. Both species exhibited initial symptoms, such as reduced growth and changes in leaf colour, which are characteristic of the individual deficiencies of nitrogen (N), phosphorus (P), and potassium (K). These symptoms were discernible to the naked eye by the 11th day following the application of stress. The deficiency of phosphorus (P) and a combination of nitrogen-phosphorus (NP) was the first visual change observed. Notably, the deficiency of all three nutrients (N, P, and K) led to the most substantial reduction in growth and the quickest manifestation of symptoms.

CHLOROPHYLL FLUORESCENCE PARAMETERS

Six chlorophyll *a* fluorescence parameters (F_V/F_M, ABS/CS_o, Di_o/CS_o, Tr_o/CS_o, Et_o/CS_o, and PI_{total}) were selected for further analysis from the measured and calculated data. These parameters were tested under both single and multiple nutrient deficiency stresses, revealing that the photosynthetic efficiency directly responded to the absence of one or more nutrients in the growth medium. The values were normalised in relation to the control group and depicted in radar plots for both maize and wheat at all time intervals (Figs. 1, 2). The mean values of the selected parameters, along with standard errors and the results of statistical analyses, are presented in Tables S1 and S2. The early changes were noted on the second day after the application of stress (57 DAS – days after sowing), where there was an increase in the average values of DI_o/CS_o while a reverse pattern was observed in the case of PI_{total}. These changes intensified over time, with both species displaying the highest value for the first parameter and the lowest value for the last parameter at the end of the experiment (75 DAS) – Figures 3 and 4. Furthermore, both species exhibited significant differences in the average values of ABS/CS_o, TR_o/CS_o, ET_o/CS_o, and F_V/F_M parameters between the control group, and single and multiple nutrient deficient groups at 63 DAS, which is still prior to the appearance of the first visual symptoms of stress. These results were further confirmed through PCA ordination. In the case of maize, the first two components explained 87.5% of the variance in the data. The first axis clearly distinguished the control and early responses from the late responses of single and multiple nutrient-deficient plants and was correlated with changes in PI_{total}. The second component (on the left side of the chart) formed two distinct groups: KP, NP, and NK deficient plants, associated with reduced electron trapping and

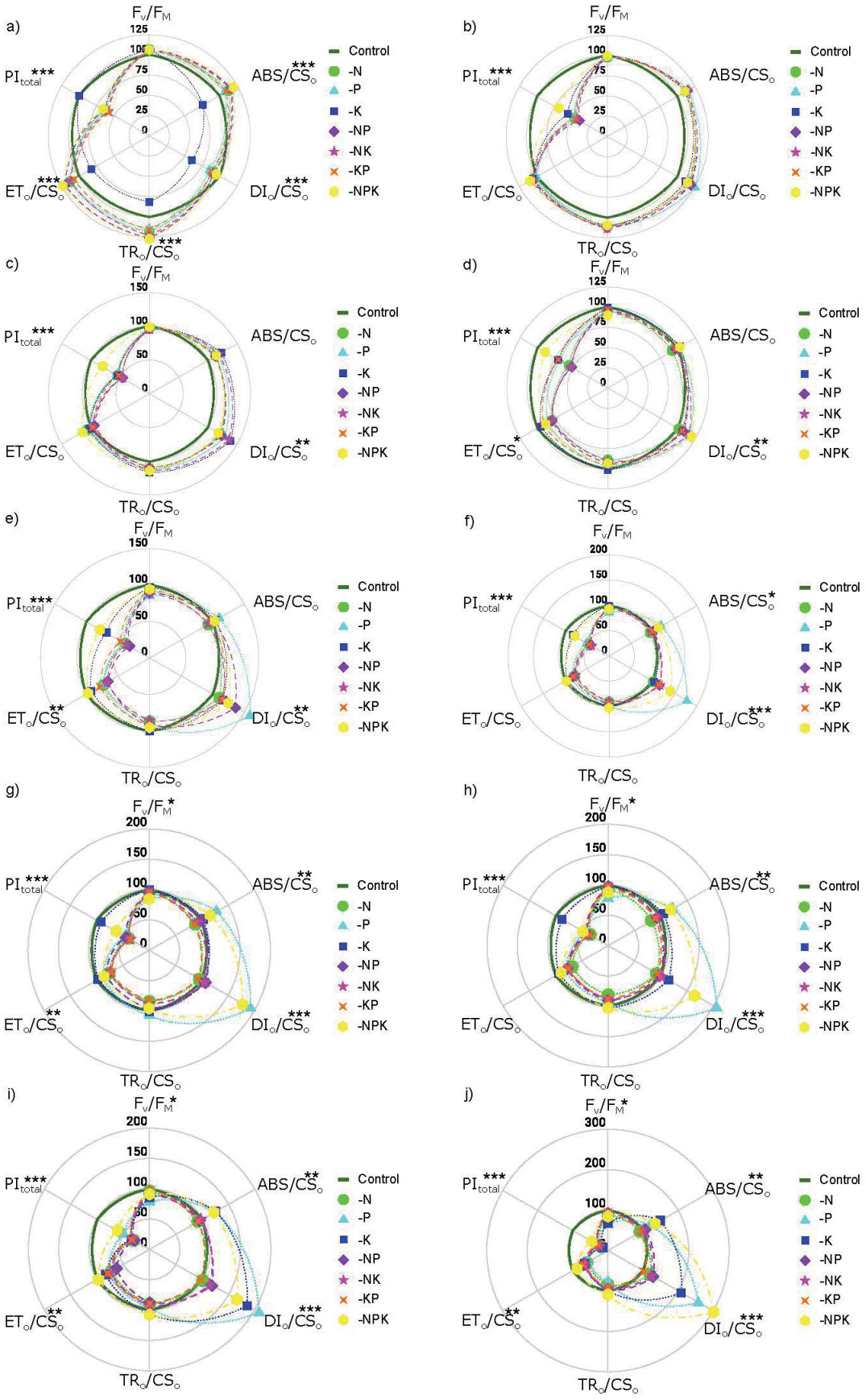

Fig. 1. JIP test parameters normalised to control plant values as radar plots for maize leaves at ten measurement time points (t_0–t_9); standard error values of the mean are given in Table S1; the asterisks denotes statistical significance of differences a chlorophyll fluorescence parameter values between control and nutrient deficiency treatments within a given time point (t) at: $p \leq 0.001$ (***), $p \leq 0.05$ (**) and $p \leq 0.01$ (*); F_V/F_M, PI_{total}, ABS/CS_o, TR_o/CS_o, DI_o/CS_o, ET_o/CS_o as in Tab. 3; source: own study

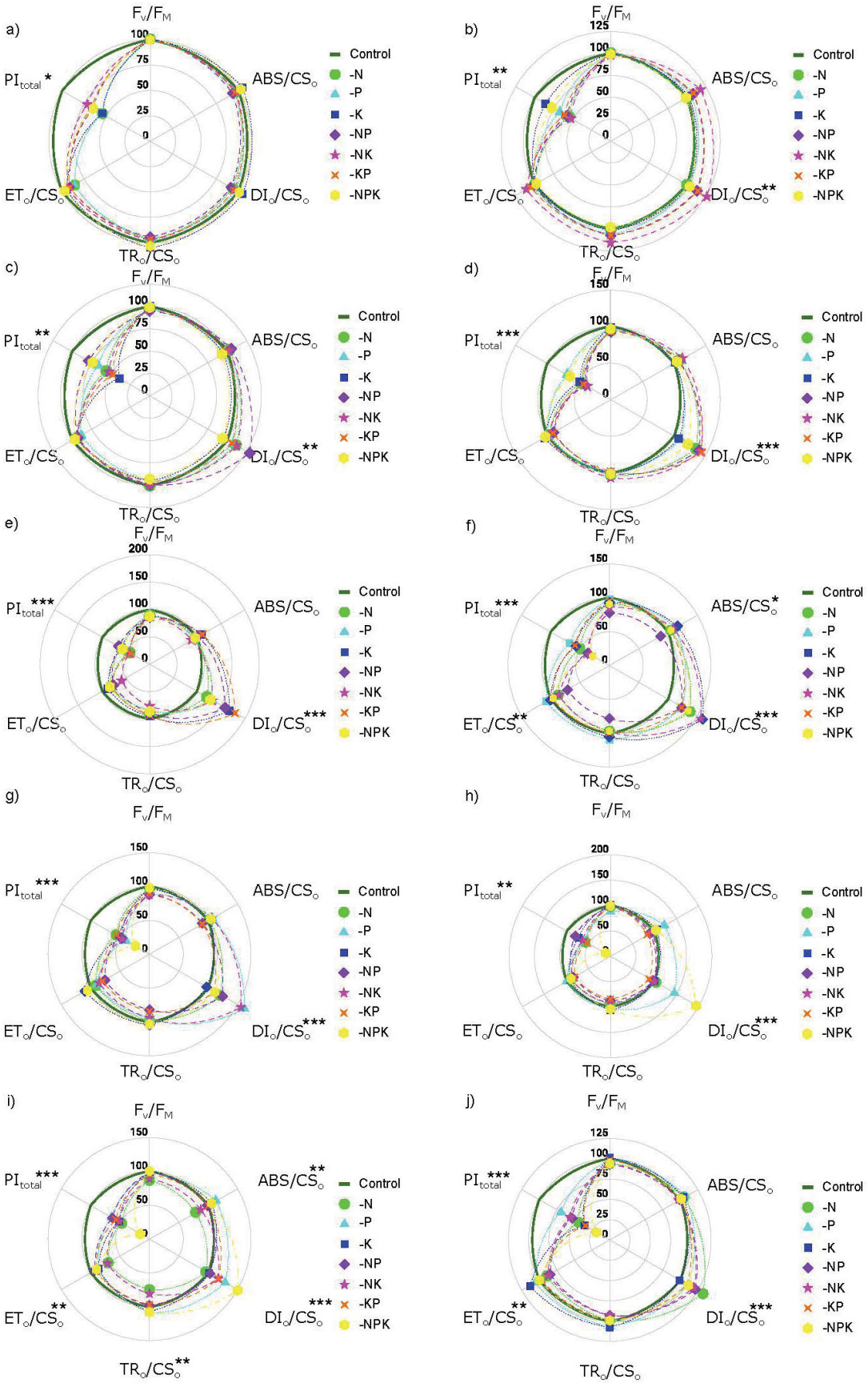

Fig. 2. JIP test parameters normalised to control plant values as radar plots for wheat leaves at ten measurement time points (t_0–t_9); standard error values of the mean are given in Table S1; the asterisks denotes statistical significance of differences a chlorophyll fluorescence parameter values between control and nutrient deficiency treatments within a given time point (t) at: $p \leq 0.001$ (***), $p \leq 0.05$ (**) and $p \leq 0.01$ (*); F_V/F_M, PI_{total}, ABS/CS_o, TR_o/CS_o, DI_o/CS_o, ET_o/CS_o as in Tab. 3; source: own study

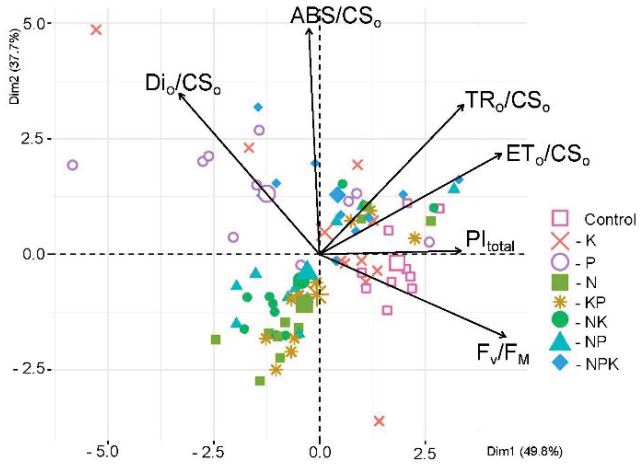

Fig. 3. Principal component analysis of six selected chlorophyll fluorescence parameters, measured during ten time steps (t_0–t_9) in maize under single and multiple nutrient deficiency; the first two components explained 87.5% of total variance in the data; F_V/F_M, PI_{total}, ABS/CS_o, TR_o/CS_o, DI_o/CS_o, ET_o/CS_o as in Tab. 3; source: own study

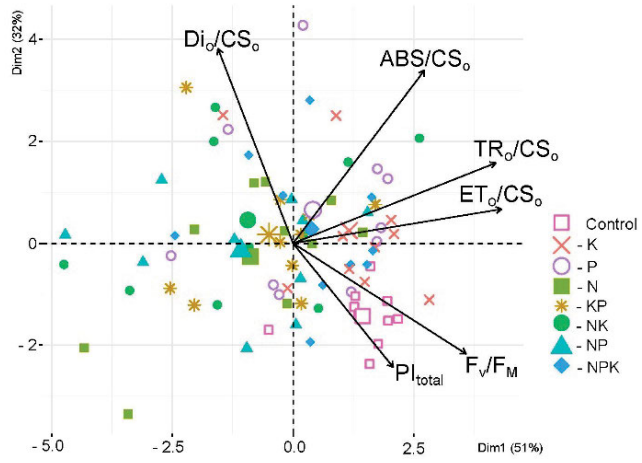

Fig. 4. Principal component analysis of six selected chlorophyll fluorescence parameters, measured during ten time steps (t_0–t_9) in wheat under single and multiple nutrient deficiency; the first two components explained 83.0% of total variance in the data; F_V/F_M, PI_{total}, ABS/CS_o, TR_o/CS_o, DI_o/CS_o, ET_o/CS_o as in Tab. 3; source: own study

transfer (TR_o/CS_o, ET_o/CS_o) and P, K, and NPK with high DI_o/CS_o and F_V/F_M (Fig. 2). The similar, but slightly less clear pattern was found in wheat (Fig. 4).

DISCUSSION

The impact of various stresses on plants have been detected using photosynthesis and chlorophyll fluorescence (ChFl). Originally, practical research applying ChFl measurements were done to assess plant responses to broad biotic and abiotic stresses. Eventually, it was used to amplify plant growth and yield, potentially increase food production and supporting agricultural sustainability. We pushed our hypothesis a step further and discovered that ChFl readings may be used to non-invasively monitor photosynthesising species in different environments and detect the sort of stress. We recently concentrated on developing a biological feedback system that allows plants to manage their development parameters, such as light quality and intensity, using artificial intelligence and machine learning. The important step to achieve this goal is to find the ChlF parameters, highly variable sensitive to changes in single and multiple nutrient content in plant tissue.

Recording OJIP fluorescence transients in our experiments, followed by analysis with the JIP test, allowed us quantification of photosynthetic parameters that provide insight into N, P, K and their combination (PK, NP, NK, NPK) deficiency-induced changes in PSII function. We detected significant differences in two parameters (PI_{total} and DI_o/CS_o) that enables us to find differentiate in maize and wheat plants under this single and multiple nutrient deficiency stress at 57 DAS i.e. long before any visual symptoms of stress appear. Parameters that decreased during the stress in both species were: TR_o/CS_o and ET_o/CS_o.

Up to date, most of the studies on relationships between chlorophyll *a* fluorescence and plant nutritional status, concentrated on effects of single nutrient deficiency. The results of Živčák *et al.* (2014), Alexandrov *et al.* (2014) and Cetner *et al.* (2017; 2020), confirmed that N, P, and K deficiency decreased total electron carriers per *RC* (EC_o/RC), yields (TR_o/ABS, F_V/F_M, ET_o/TR_o, RE_o/ET_o, ET_o/ABS and RE_o/ABS), fluxes (RE_o/RC and RE_o/CS_o) and fractional reduction of PSI end electron acceptors, and damaged all photochemical and non-photochemical redox reactions as indicated by decreases in PI_{abs} and PI_{total}. This implies that plants under single nutrient deficiency stress have a decreased capacity of electron transport. This means that ATP synthesis and Ribulose 1,5-bisphosphate regeneration are blocked. Some of rare studies, performed on natural or field seems to confirm obtained results. Kalaji *et al.* (2018b) studied response of rapeseed to different nutrient status in in natural soil with JIP test, PCA and machine learning methods (super self-organising networks, sSOM). They found strong differences among others in maximum quantum efficiency of photosystem II (φ_{Po}) and PI_{total} parameters. However under natural conditions, micro- and macronutrients are present in different concentrations and forms, and in varying availability to the plants which often precludes the possibility to study explicitly the simple and combined effects of micro- and macronutrients on photosynthetic efficiency. Horaczek *et al.* (2020) on *Miscantus* sp. studied plant response to nutrient status. They found differences between control and the plants from NPK, in DI_o/CS_o parameter which results are in line with our study.

We conclude that JIP test parameters together with application of advanced multivariate techniques, such as principal component analysis, should be considered very useful for stress identification (Goltsev *et al.*, 2012; Kalaji *et al.*, 2014b; Kalaji *et al.*, 2017b).

AUTHOR CONTRIBUTIONS

1st Author (contribution – 70%): study design and performance of the experiments, data collection, manuscript preparation and literature search. 2nd Author (contribution – 30%): statistical analysis, data interpretation and manuscript preparation.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material to this article can be found online at https://www.jwld.pl/files/Supplementary_material_Jaszczuk.pdf

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