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Early Detection of Plant Stress from Changes in Distributions of Chlorophyll *a* Fluorescence Parameters Measured with Fluorescence Imaging

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Abstract In this work, we used barley leaves suffering from a stress, for measurements of chlorophyll *a* fluorescence with an imaging fluorometer. We compared selected fluorescence parameters (FP) determined from the measurements of control (no stress) and afterwards stressed sample by classical statistical comparison (Mann–Whitney test) and by statistical comparison of shapes of distributions of the FPs (two-sample Smirnov test). We have found that there exist examples where statistically significant difference is not revealed using the classical statistical comparison (for given critical level), but statistically significant difference is revealed using comparisons of distributions (for the same critical level). It implies that the shape of statistical distribution of a FP is more sensitive to a stress of a sample than median of the FP. Further, the comparison of changes in shapes of statistical distributions of FPs is therefore more suitable for early detection of plant stress than a classical statistical comparison. The observed changes in the distributions of FPs are discussed.

Keywords Chlorophyll · Fluorescence · Imaging · Photosynthesis · Statistics · Stress

Introduction

Measurements of chlorophyll *a* fluorescence induction (FI), also called Kautsky effect [1], is a widespread method to access the function of photosynthetic apparatus (for reviews see [2,3]). FI represents a time course of fluorescence emitted by a sample illuminated by continuous light and it consists of a fast fluorescence rise (FLR) followed by a slow fluorescence decrease. While the decrease in the FI reflects fluorescence quenching by non-photochemical reactions, the FLR informs us about photochemical events (light-driven electron transport) occurring among electron carriers placed in thylakoid membrane of chloroplast.

The FI is usually measured with conventional (i.e. non-imaging) fluorometers, where an integral signal coming from a measuring spot of a sample is detected. To access a stress action, several samples must be used to obtain a suitable amount of data for subsequent statistical testing. Results of such statistical testing then represent how a population of samples (different leaves or even different plants) response to a stress action. However, usage of imaging fluorometers enables to obtain a large amount of data even from one sample (one leaf) and therefore to study a stress action to one sample and not to a population of samples.

Fluorescence parameters (FPs) determined from the FI are usually presented in literature by means of the mean and standard deviation (or standard error) (e.g. [4]). But the above presentation of data "correctly" describes data with symmetrical distributions (best with the Gaussian distribution). However, we found, using a conventional fluorometer, that FPs determined from the FLR generally do not have Gaussian (or symmetrical) distributions of data and that the variance and shape of distribution of given FP is changing upon stress of the samples [5–7]. Therefore, the usage of data presentation by means of the mean and standard deviation (or standard error) is not appropriate and it masks the real data distribution of the FPs. Using of median, quartiles and maximal and minimal values describes better a real situation of data distribution [5]. Further, because the parameters of the FLR generally do not have Gaussian distributions, the non-parametric test should be used for statistical comparisons of the parameters rather than the parametric tests, which

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are based on assumption of Gaussian distribution of data [e.g. 8,9].

In this work, we used an imaging fluorometer to study statistical properties of the FPs upon a stress action on the sample. We found that stress-induced changes in statistical distributions of the FPs occur earlier than stress-induced changes in medians of the FPs.

Materials and methods

Spring barley (*Hordeum vulgare* L. cv. Akcent) plants were cultivated for 9 (younger plants; growth phase 12 according to [10]) and 14 days (older plants; growth phase 13 according to [10]) in a light / dark regime (16 hr light / 8 hr dark; 90 μmol photons m^{-2} s^{-1} of photosynthetically active radiation) in a growth chamber in artificial soil composed of perlit and Knop solution at 23°C. A tip (~1.5 cm) was detached from the primary leaf blades and about 5 cm long segments of the leaf blades were used for measurements.

Two segments (one from the older and one from the younger plants) were fixed side by side on a plate and kept at room temperature in darkness for 10 min and chlorophyll fluorescence was then measured. This measurement served as the control. After the measurement, the segments were kept in their fixed positions in darkness and the measurement was repeated every 60 min. This routine was used to cause a stress to the photosynthetic apparatus mainly by a dehydration of the segments. Such treatment was used for the purpose, to obtain only small changes in the FPs caused by stress action to detect expected statistical properties of the FPs.

Fluorescence signal was excited and detected from adaxial side of the leaf blade segments and the measurements were performed in darkness by kinetic imaging fluorometer FluorCam 700MF (Photon Systems Instruments Ltd., Brno, Czech Republic) described in detail in [11]. Briefly, fluorescence was measured during 10 μs long measuring flashes ($\lambda = 650$ nm) generated by two panels of light-emitting diodes (LED). The same LED panels also provided continuous actinic light of intensity of 120 μmol photons m^{-2} s^{-1}. Saturating white pulse of intensity of 900 μmol photons m^{-2} s^{-1} was provided by 250 W halogen lamp. Minimal fluorescence F_0 was taken as fluorescence signal initiated by measuring flashes placed 1 s apart in dark with a dark-adapted plant material. Maximal fluorescence F_M (dark-adapted state) was measured in the middle of 1.6 s long saturating pulse. Fourty second after the end of saturating pulse, continuous actinic light was switched on for 62 s to cause FI. A peak fluorescence (F_P) measured during the FI and terminal fluorescence at the end of the FI (designated here as steady-state fluorescence; F_S) were evaluated. In addition to basic fluorescence parameters mentioned above, following FPs were calculated: $(F_M - F_0) / F_M = F_V / F_M$ which expresses maximal quantum yield of photosystem II (PSII) photochemistry [12], $(F_M - F_0) / F_0 = F_V / F_0$ which expresses a fraction of functional PSII reaction centres [13], a ratio F_M / F_0, and the vitality index R_{fd} ($= (F_P - F_S) / F_S$) [14].

The fixed position of the segments and usage of a mask in FluorCam software enabled the fluorescence signal to be evaluated always from the same segment area. Each segment provided about 1300 datapoints (pixels) for statistical analysis. In the analysis, given FP of the control was compared with the FP of stressed sample after different durations of the stress. As statistical distributions of the FPs generally are not Gaussian (see Introduction, but also tested here by the Kolmogorov–Smirnov test), the Mann–Whitney test was used for comparison of the medians of given FP. To compare changes in a given FP on the basis of the shapes of its statistical distributions, the two-sample Smirnov test was used. Two-sided p-values of the tests were evaluated. SigmaStat (SPSS Inc., Chicago, USA) and StatsDirect (StatsDirect Ltd., Cheshire, UK) were used for the statistical calculations. If not mentioned otherwise, the critical level α for all used statistical tests was chosen to be 0.01, which means that if the p-value of a test is higher than α then there is not a statistically significant difference and if the p-value of a test is smaller than α then there is a statistically significant difference.

Results and discussion

We performed the measurements with leaves of different age suffering from the stress (see Materials and methods) several times and the results were, in general, the same. Therefore, results of one set of measurement are presented here. Examples of FI curves measured with the controls are shown in Fig. 1A. Fluorescence signal at each point of given FI curve represents an integral value over the whole leaf blade segment. However, values of fluorescence signal coming from all pixels over whole leaf blade segment can be obtained at each point of given FI curve. Therefore, histograms of any basic (F_0, F_M, F_P, F_S) or ratio (F_V / F_M, F_V / F_0, F_M / F_0, R_{fd}; see Materials and methods) FP can be constructed and used for subsequent statistical analysis. For example, histograms of maximal quantum yield of PSII photochemistry (the F_V / F_M ratio) of the controls are shown in Fig. 1B.

The histograms of Fig. 1B confirm our previous results that FPs determined from the FLR measured using a conventional fluorometer do not have Gaussian distributions of data and that the distributions are generally skewed to a side (skewed to the left in case of Fig. 1B) [5–7]. However, Fig. 1B shows that this fact is valid not only for a population of samples as found in [5–7] but is also inherent for one sample. The

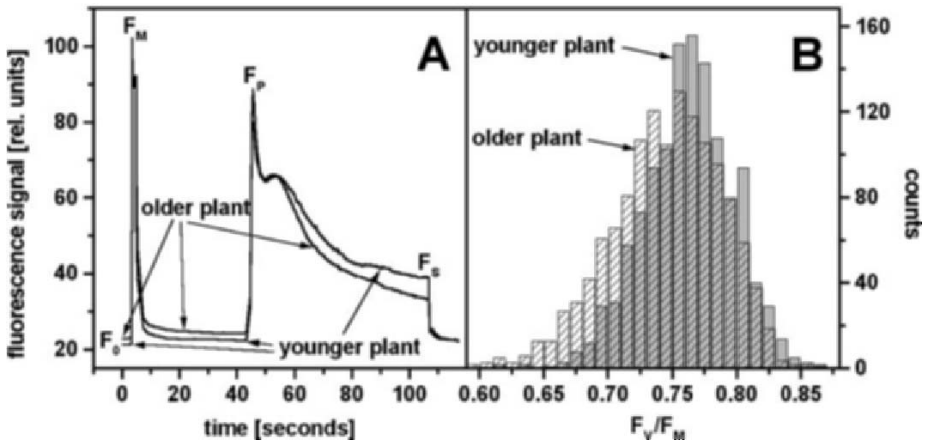

Fig. 1 (A) Examples of measured FI curves (see Materials and methods for the measuring protocol) with the controls of older (14 days) and younger (9 days) plants. The F_0, F_M, F_P and F_S levels are marked. (B) Distributions (histograms) of the F_V/F_M ratio of the controls of the older and younger plants

non-Gaussian distributions of data, even visually presented in Fig. 1B (but also tested by Kolmogorov–Smirnov test for all the FPs; data not shown), also indicate a necessity of usage of the non-parametric tests (they are not based on the assumption of Gaussian distribution of data) for statistical comparisons of the FPs.

Simple visual comparison of the FI curves from Fig. 1A and of the histograms from Fig. 1B shows that there is a difference between the younger and older controls and therefore that age of the plant material is reflected in the FI curves and corresponding FPs. To be more rigorous, statistically significant difference between the controls was found using Mann–Whitney test for all the basic and ratio FPs (p-value$<$ 0.0001), except of F_M (p-value $=$ 0.1555).

The main goal of this work was to find examples where the stress action (dehydration in our case; see Materials and methods) will not lead to a statistically significant difference between medians of a FP of the control and afterwards stressed leaf blade segment (tested by Mann–Whitney test), but the stress action will cause a statistically significant difference between distributions (histograms) of the FP of the control and afterwards stressed leaf blade segment (tested by two-sample Smirnov test). The FPs for which we found such behaviour are summarised in Table 1. Cases where the p-value of Mann–Whitney test (the first numbers) was higher than the critical level α (i.e. there is no statistically significant difference between medians of the FP of the control and afterwards stressed leaf blade segment) and, at the same time, the p-value of two-sample Smirnov test (the numbers in parentheses) was lower than α (i.e. there is a statistically significant difference between distribution of the FP of the control and afterwards stressed leaf blade segment). For the other FPs or longer stress action on the FPs in Table 1, a statistically significant difference between medians of FP of the control and afterwards stressed leaf blade segment was found.

The results of Table 1 clearly show that stress action causes a statistically significant difference in distributions of FPs while the medians of the FPs are not significantly changed. In the data presented in Table 1 we even found one case (the F_V / F_0 ratio for segment from the older plant) where the median had the same value for the control and afterwards stressed leaf blade segment, but there was a statistically significant difference in the distributions. This is for illustration presented in Fig. 2. This result demonstrates that the assessment of stress action by statistical comparison based on changes in medians need not lead to a correct conclusion as for a treatment-induced stress on a sample and therefore masks real situation occurring in the sample. The results of Table 1 and Fig. 2 also imply that the shape of statistical distribution of a FP is more sensitive to a stress of a sample than median of the FP and that the comparison of

Table 1 FPs and statistically significant differences at different durations of stress action on younger and older plant segments.

	Younger plant		Older plant	
FP	1 hr	2 hr	1 hr	2 hr
F_0	0.2957[a] (<0.0001)[b]	0.0502 (<0.0001)	–	–
F_V / F_M	0.0174 (<0.0001)	–	0.1356 (0.0249*)	–
F_V / F_0	0.0207 (<0.0001)	–	0.1625 (0.0009)	–
F_M / F_0	0.0208 (<0.0001)	–	0.1113 (0.0138*)	–
F_P	–	–	0.0188 (<0.0001)	0.0238 (<0.0001)

Note. α was chosen to be 0.01 except of the cases denoted by asterisks where α was chosen to be 0.05. The dashes indicate that a statistically significant difference between medians of the FP of the control and afterwards stressed leaf blade segment was found.

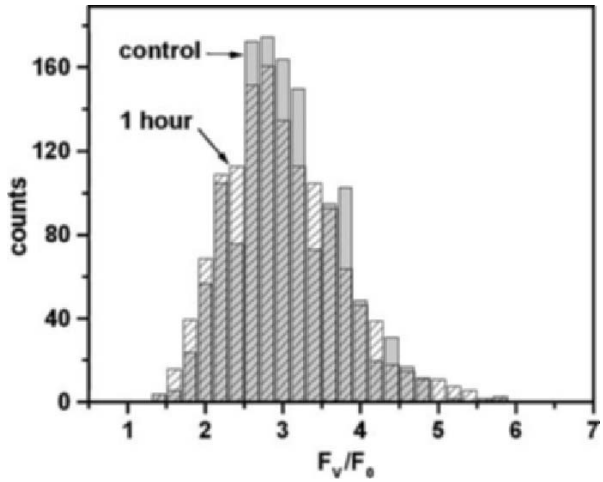

Fig. 2 Distributions (histograms) of the F_V/F_0 ratio of the older (14 days) plant measured for the control and after the stress treatment for 1 hr. The medians were in both cases the same (2.9569) and there was no statistically significant difference between the medians (p-value of Mann–Whitney test = 0.1625), but there was a statistically significant difference in the distributions (p-value of two-sample Smirnov test = 0.0009)

changes in shapes of statistical distributions of FPs is more suitable for early detection of plant stress than using classical statistical comparison.

A stress action is usually accompanied by an increase in the F_0 and F_S values and by a decrease in F_M and F_P values that results in a decrease of F_V/F_M, F_V/F_0, F_M/F_0, and R_{fd}. Therefore, when a median of a FP is not significantly changed, as found by us for some FPs, a 'correct' stress-induced change of the FP should be "equilibrated" by an opposite change of the FP (see Fig. 2). It means that not all photosynthetic units respond to a stress treatment by expected behaviour and that the stress treatment can even result in better function in some photosynthetic units. Therefore, environmental conditions for optimal photosynthetic function can be different even within one sample.

In conclusion, our work shows that fluorescence measurements by an imaging fluorometer offer a deeper insight into the statistical properties of FPs. Although our results show that the evaluation of changes in shapes of distributions of the FPs is more suitable for early detection of dehydration stress on photosynthetic material than the evaluation of changes in medians of the FPs, further work is needed so that this approach will become a routine in assessment of stress action in photosynthesis research generally.

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