Statistical properties of chlorophyll fluorescence induction parameters

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Abstract

Parameters of the fast chlorophyll (Chl) fluorescence induction (the O-J-I-P curve) of plants of winter wheat grown in the field canopy were statistically tested for Gaussian distribution. Five different statistical methods showed that the obtained values did not obey the Gaussian distribution law. The presentation of the parameters with the help of the mean and standard deviation masks the information about statistical properties of the values. Thus, we recommend to present the parameters by means of median, quartiles, and minimum and maximum values rather than by means of the mean and standard deviation.

Additional key words: Gaussian distribution; leaf age; *Triticum aestivum*; wheat.

Introduction

Chl fluorescence induction is a widespread method in photosynthesis research. The main parameters F_0, F_M, and F_V/F_M are mostly presented using the mean and standard deviation with relatively low number of measurements. The Gaussian (also called normal) or other symmetrical distribution should be expected when inspecting the results. However, the parameters of Chl fluorescence induction are characterised by upper or lower limiting values, and this may influence the symmetry of the data distribution. In an asymmetrical sample of values the mean and standard deviation may induce misleading conclusions. Further, the normal distribution of values is one of the conditions (besides equality of variance) for using parametric tests for statistical testing.

In this paper we concentrate on the characterisation of statistical properties of Chl fluorescence induction values (199 measurements), particularly on the symmetry of distribution, and their change with the age of the leaves of wheat plants in a canopy.

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Materials and methods

Winter wheat (*Triticum aestivum* L. *cv.* Siria) was grown in a field in a canopy (550 plants per m^2) under natural conditions (near Kroměříž, Moravia) in the years 1993-1994. The plants (June 23, 1994) were in growth phase 71 DC according to Zadoks (watery grain - see Zadoks *et al.* 1974). There were four leaves on the stalk distinctly discernible below the spike. The first leaf, counted from the apex (the flag leaf), could bear some indications of photoinhibition (results not shown). On the contrary, the fourth leaf situated in the lowest level of the canopy showed patterns of strong senescence and illness (yellow colour, drying). For these reasons we have chosen the second and the third leaf from the apex for the measurements. The second leaf had the best visual and fluorescence characteristics (fully green, F_V/F_P value above 0.8) whereas the third leaf was already senescing. The mean sunlight irradiance of the third leaf was about one half of the irradiance of the second leaf (measured with quantum radiometer *LI-189, LI-COR*, USA).

Chl fluorescence induction curves were measured using the Plant Efficiency Analyser (PEA, *Hansatech*, England) with red irradiance of 3000 μmol m^{-2} s^{-1} and the maximum at about 650 nm. Fluorescence signal was detected from the adaxial side of the central part of the leaf blade. The leaves were predarkened in clips for 30 min. Time of fluorescence registration was 2 s.

Statistical testing of normality of data distribution was performed using the Kolmogorov-Smirnov test with Lilliefors' correction, the D'Agostino's test, the Shapiro-Wilk test, and by calculation of skewness and kurtosis. The results were calculated using the spreadsheet in program *Excel* version *5* (*Microsoft Corporation*, USA). For the theory of all methods and statistical evaluations see, *e.g.*, Kotz *et al.* (1989), Triola (1989), Motulsky (1995), and Sprinthall (1996).

For practical reasons, we used our own graphical presentation of values which we call Point-and-Whiskers Plot. It has the same meaning as the well-known Box-and-Whisker Plot: the point means median, the whisker nearer to the point means 25 and 75 % quantiles, and the whisker farther from the point means minimum and maximum values. As for the normal distribution, the interval between bars of standard deviations contains about 68 % of all data. That is why we present in Fig. 2 the same interval by means of 16 and 84 % quantiles designated by asterisks which show a real distribution of the values.

Results and discussion

A typical Chl fluorescence induction curve measured with a leaf blade is shown in Fig. 1. Particular steps O, J, I, and P are marked. Positions of the J and I steps were at 2 and 20 ms, respectively. The minimal fluorescence intensity F_0, the intensities at J (F_J) and I (F_I) steps, and maximal fluorescence intensity F_P were evaluated. These quantities were used for calculating variable fluorescence $F_V = (F_P - F_0)$, relative variable fluorescence of the J and I steps designated as $rF_J = (F_J - F_0)/(F_P - F_0)$ and $rF_I = (F_I - F_0)/(F_P - F_0)$, respectively, and maximum quantum yield of photosystem 2 photochemistry F_V/F_P. For more information about FIC see, *e.g.*, Strasser and Govindjee (1992a,b) or Govindjee (1995).

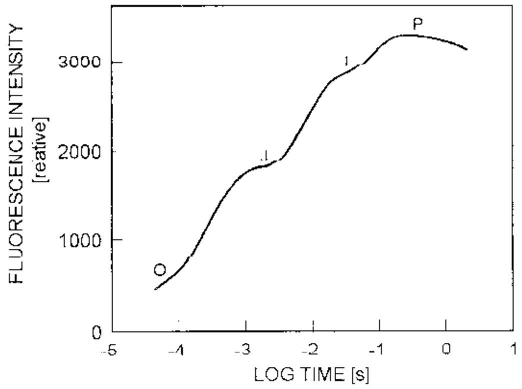

Fig. 1. A typical chlorophyll fluorescence induction curve measured with wheat leaves using the Plant Efficiency Analyser with marks of particular steps.

Before presentation of our results we want to give a brief description of the statistical theory involved in this work. The first thing is formulation of the so-called null hypothesis which means in our case that there is no difference between experimental data and Gaussian (normal) distribution. The statistical testing tells whether the null hypothesis can be accepted or rejected. But even if we accept (or reject) the null hypothesis, there is always a probability that our decision is wrong. This probability is called significance level α, and it is the highest acceptable probability of incorrect concluding that there is a difference. In our testing we chose a usual value $\alpha = 5$ %. Further, there is a number called critical value which is assigned to significance level in dependence on the chosen test and number of values. The result of the particular test after its application to the experimental values is a number which is called test statistics. In this work we used the Kolmogorov-Smirnov, D'Agostino's, and Shapiro-Wilk tests whose test statistics are usually marked as K-S distance, Y, and W, respectively. The theory of these tests implies that the null hypothesis can be excepted (the population obeys normal distribution; denoted as Y in Tables 2 and 3) if the following relations hold:

$$\text{K-S distance} < (\text{K-S})_\alpha \text{ distance}$$

for the Kolmogorov-Smirnov test,

$$Y_{\alpha/2} < Y < Y_{1-\alpha/2}$$

for the D'Agostino's test, and

$$W > W_\alpha$$

for the Shapiro-Wilk test. Otherwise the null hypothesis must be rejected (the population does not obey normal distribution; denoted as N in Tables 2 and 3). The letters with subscript α are the critical values for the particular tests at the level of significance α. The critical values for the tests in dependence on number of values in population are presented in Table 1. For further statistical testing we evaluated skewness and kurtosis as measures of the symmetry of the distribution and the shape of distribution curve, respectively. Both skewness and kurtosis equal zero for normal distribution.

Table 1. Critical values for the particular tests (see Results and discussion) in dependence on number of values in population. The critical values are valid for significance level α = 5 %.

Used test	Kolmogorov-Smirnov test	D'Agostino's test				Shapiro-Wilk test	
Number of values	199	100		32		30	7
	$(K\text{-}S)_\alpha$ distance	$Y_{\alpha/2}$	$Y_{1-\alpha/2}$	$Y_{\alpha/2}$	$Y_{1-\alpha/2}$	W_α	W_α
Critical value	0.0635	-2.54	1.31	-2.88	0.862	0.927	0.803

We measured the FICs of 199 pieces of the second as well as the third leaves from the apex and we tested the null hypothesis that the distribution of values is of the Gaussian type. Besides of the skewness, kurtosis, and Kolmogorov-Smirnov test which were used for testing of all 199 values of all the fluorescence parameters and both leaves, there were some restrictions as for other two tests. The D'Agostino's test is designed for testing of normality of populations containing from 31 to 100 data sample. Thus, we have chosen 32 and 100 values from the group of 199 values randomly, and we tested them for their normality. The Shapiro-Wilk test is designed for testing of a population containing from 7 to 30 data sample. We have chosen 7 and 30 values from the whole population randomly, and we applied this test.

The values of test statistics for the used statistical methods are presented for the second and third leaf from the apex in Tables 2 and 3, respectively. The results of statistical testing (after a comparison of the test statistics in Tables 2 and 3 with the critical values in Table 1) are also presented.

Except for the Kolmogorov-Smirnov test for the second leaf from the apex (Table 2), all other four statistical methods found that none of the fluorescence parameters obeyed the Gaussian distribution. The Kolmogorov-Smirnov test detected normal distribution of the F_J, F_I, F_P, rF_I, and F_V parameters of the second leaf. This test probably did not distinguish the asymmetry of distributions due to a small number of values (the test is designated for up to 2000 values).

As for the third leaf from the apex (Table 3), all the used statistical methods showed that all the parameters did not obey the normal distribution of values. The asymmetry of F_J, F_I, F_P, rF_I, and F_V parameters increased so that even the Kolmogorov-Smirnov test revealed only asymmetrical distributions.

A presentation of the asymmetrically distributed values with the help of the mean and standard deviation may be unsuitable because these statistical parameters are of clear meaning only for the Gaussian distribution. The presentation by Point-and-Whiskers Plot (see Materials and methods) is more informative in such a case. To illustrate the difference in the two statistical presentations, we show our results (Fig. 2) with values plotted by means of the mean and standard deviation as well as by means of the Point-and-Whiskers Plot. Further we show 16 and 84 % quantiles designated by asterisks (for the reason see Materials and methods).

Table 2. Values of test statistics for the particular statistical method (see Results and discussion) and the particular parameters determined from FIC for the second leaf from the apex of wheat plant. Y - acceptation of the null hypothesis (the population obeys normal distribution), N - rejection of the null hypothesis (the population does not obey normal distribution). F_0, F_P, F_J, and F_I are minimal and maximal fluorescence intensities, and fluorescence intensities at the J and I steps, respectively. F_V, F_V/F_P, rF_J, and rF_I are variable fluorescence, maximum quantum yield of photosystem 2 photochemistry, and relative variable fluorescence of the J and I steps, respectively.

Statistical method	Kolmogorov -Smirnov test	D'Agostino's test		Shapiro-Wilk test		Skewness	Kurtosis
Number of values	199	100	32	30	7	199	199
F_0	0.0651; N	-110.27; N	-62.10; N	0.164; N	0.269; N	0.789; N	2.664; N
F_J	0.0516; Y	-100.43; N	-58.73; N	0.025; N	0.278; N	0.322; N	0.433; N
F_I	0.0565; Y	-108.77; N	-67.40; N	0.078; N	0.295; N	-0.109; N	0.433; N
F_P	0.0543; Y	-113.81; N	-68.29; N	0.121; N	0.287; N	-0.346; N	0.982; N
rF_J	0.0816; N	-80.79; N	-46.73; N	0.015; N	0.210; N	0.401; N	0.814; N
rF_I	0.0528; Y	-91.91; N	-60.82; N	0.000; N	0.257; N	-0.253; N	-0.121; N
F_V	0.0607; Y	-113.40; N	-68.32; N	0.108; N	0.282; N	0.497; N	1.026; N
F_V/F_P	0.0892; N	-104.47; N	-62.11; N	0.004; N	0.001; N	-1.493; N	5.588; N

Table 3. Values of test statistics for the particular statistical method (see Results and discussion) and the particular parameters determined from FIC for the third leaf from the apex of wheat plant. N - rejection of the null hypothesis (the population does not obey normal distribution). Meaning of the parameters is the same as in Table 2.

Statistical method	Kolmogorov -Smirnov test	D'Agostino's test		Shapiro-Wilk test		Skewness	Kurtosis
Number of values	199	100	32	30	7	199	199
F_0	0.1739; N	-107.23; N	-62.95; N	0.045; N	0.001; N	1.656; N	4.545; N
F_J	0.1621; N	-100.97; N	-54.75; N	0.001; N	0.126; N	-1.562; N	2.586; N
F_I	0.1361; N	-97.58; N	-55.20; N	0.008; N	0.004; N	-1.175; N	1.241; N
F_P	0.1190; N	-96.83; N	-55.88; N	0.009; N	0.022; N	-1.066; N	0.955; N
rF_J	0.0889; N	-98.51; N	-48.50; N	0.007; N	0.241; N	-0.409; N	-0.636; N
rF_I	0.0652; N	-101.96; N	-45.67; N	0.001; N	0.004; N	-0.501; N	-0.132; N
F_V	0.1077; N	-93.94; N	-53.41; N	0.023; N	0.024; N	-0.901; N	0.247; N
F_V/F_P	0.2103; N	-87.97; N	-49.57; N	0.048; N	0.004; N	-2.349; N	6.873; N

The Chl fluorescence parameters of the second leaf show a rather narrow distribution of values with low standard deviations (Fig. 2*A*). Both statistical presentations seem to be equivalent, and only a deeper inspection of values reveals some asymmetrics (*e.g.*, values of F_0 and F_V/F_P). The second leaf of the studied wheat plants was in the best functional situation, screened partly by the flag leaves from the direct sun irradiation, and without indications of stress or senescence (the F_V/F_P value was in the optimal range). This state seems to be characterised not only by the optimal mean values but also by a narrow and nearly symmetrical statistical distributions of the measured values.

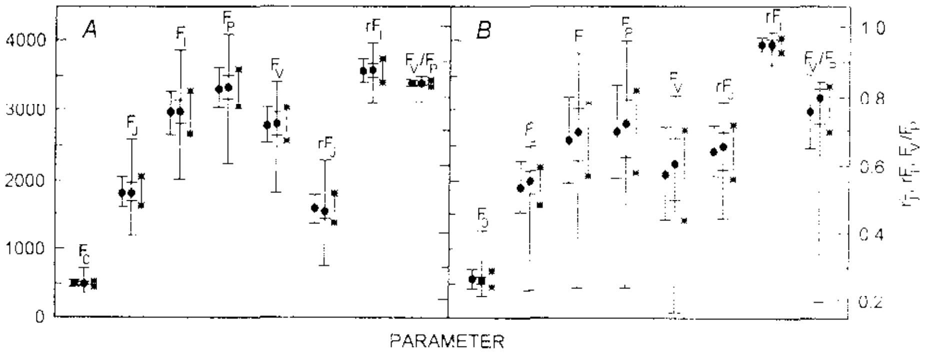

Fig. 2. The presentations of values using the mean and standard deviation (on the left side of each trinity) and Point-and-Whiskers Plot (in the middle of each trinity) for the chlorophyll fluorescence parameters for the second (*A*) or third (*B*) leaf from the apex of wheat plant. Asterisks denote 16 and 84 % quantiles (on the right side of each trinity). For an explanation of the Point-and-Whiskers Plot and 16 to 84 % range see Materials and methods.

However, the situation of the third leaf (Fig. 2*B*) was different. The mean values of rF_J and rF_I were substantially higher than for the second leaf whereas the values of F_P, F_V, and particularly F_V/F_P were lower. The standard deviations were much higher indicating a higher scatter of the measured values. In most cases, the bars of standard deviations marked a wider interval than the interval of 68 % of measured values (*asterisks*). The distributions of values were clearly asymmetrical in this case. Except for the F_0 quantity, the asymmetry was expanded in direction of lower values. The asymmetrical effect is caused by a limitation of the estimated values from the upper (all parameters except F_0) or lower (F_0) side. This caused the medians to be higher than the mean values (except F_0). The third leaf of the studied plants was apparently loosing its functional state due to senescence and stresses (low irradiance, illness). Some stresses are known to increase the J step (Strasser and Govindjee 1992a) and to decrease the F_V/F_P value and/or to cause an F_0 increase (Schreiber and Armond 1978, Klinkovsky and Naus 1994, Lichtenthaler 1996). The senescence or the reaction to the stress are characterised also by a higher scatter of the measured values. The plants in canopy and the leaf tissue develop the senescence symptoms individually and in a heterogeneous way. These facts are documented also by the Point-and-Whiskers Plot (Fig. 2*B*).

A striking example of an unsuitable presentation of the measured values with the help of the mean and standard deviation is the F_V/F_P parameter in Fig. 2*B*. The upper bar of the standard deviation indicates values higher than the real maximum of the measured values.

In conclusion, we suggest to present the statistical data of Chl fluorescence induction measurements in the form of Point-and-Whiskers Plot rather than by means of the mean and the standard deviation. Presentation by the Point-and-Whiskers Plot better describes the real situation and reveals the asymmetry of values which increases with increasing stress of plants.

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