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Chloroplast functionality assessment by flow cytometry: Case study with pea plants under Paraquat stress

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Abstract

Photosynthesis is one of the most important processes in plant biology and in the development of new methodologies that allow a better understanding and characterization of the photosynthetic status of organisms, which is invaluable. Flow cytometry (FCM) is an excellent tool for measuring fluorescence and physical proprieties of particles but it has seldom been used in photosynthetic studies and thus the full extent of its potentialities, in this field of research, remains unknown. To determine the suitability of FCM in photosynthesis studies, pea plants were exposed to Paraquat and their status was analyzed during 24 h. FCM was used to evaluate the integrity (volume and internal complexity) and the relative fluorescence intensity (FL) of chloroplasts extracted from those plants. To elucidate which type of information the FL conveys, FL values were correlated with the minimum fluorescence level (F_0), maximum fluorescence level (F_m) and maximum photochemical efficiency of PSII (F_v/F_m), obtained by using Pulse-Amplitude-Modulation (PAM) fluorometry. Results indicate that: (*1*) the biomarkers used to evaluate the structural integrity of the chloroplasts were more sensitive to Paraquat exposure than the ones related to fluorescence; (*2*) the variation of the chloroplast's structure, as time progressed, pointed to a swelling and subsequent burst of the chloroplast which, in turn, compromised fluorescence emission; (*3*) FL presented a high and significant correlation with the F_v/F_m and to a lesser degree with F_m but not with F_0; (*4*) pigment content did not reveal significant changes in response to Paraquat exposure and is in agreement with the proposed model, suggesting that the cause for fluorescence decrease is due to chloroplast disruption. In sum, FCM proved to be an outstanding technique to evaluate chloroplastidal functional and structural status and therefore it should be regarded as a valuable asset in the field of photosynthetic research.

Additional keywords: chlorophyll fluorescence; chloroplast; flow cytometry; herbicide; Paraquat; photosynthesis; pulse-amplitude modulation fluorometry.

Introduction

Among the many aspects regarding photosynthetic processes, those producing stress to plants generate major concerns. Heavy metals, organic pollutants or drought stress, to name a few, can affect diverse steps of photosynthesis (*e.g.* Azevedo *et al.* 2005, Vernay *et al.* 2007, Hattab *et al.* 2009, Váňová *et al.* 2009, Dias and Brüggemann, 2010) reducing crop yield, causing deleterious effects to ecosystems and eventually affecting human health. It is therefore important to develop new methodologies to study the photosynthetic status of organisms. These methodologies should provide new biomarkers to rapidly, easily and accurately assess plant status, facilitating the data collection process, which would be otherwise, achieved through laborious and time-consuming procedures.

Among the various techniques available to study photosynthesis, variable chlorophyll (Chl) fluorescence, mainly through PAM fluorometry (Schreiber *et al.* 1986,

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Abbreviations: Chl – chlorophyll; F_0 – minimum fluorescence level of dark-adapted state; FCM – flow cytometry; F_m – maximum fluorescence level of dark-adapted state; FL – relative fluorescence intensity; FS – forward light scatter; F_v/F_m – maximum photochemical efficiency of PSII; HS – Hepes-Sorbitol buffer; LED – light-emitting diode; PS – photosystem; Pop – population; PAM – Pulse Amplitude Modulated; SS – side light scatter.
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Maxwell and Johnson 2000) became widely used as a rapid and sensitive method to quantify *in vivo* the performance of the photosynthetic apparatus, including evaluation of photoinhibitory and pollutants effects (*e.g.* Marwood *et al.* 2000, Juneau *et al.* 2001, Ali *et al.* 2006). Because the study of phytotoxicity at the photosynthetic level has been carried out for a long time, most of the parameters measured are well established and the techniques used for these evaluations are a part of the classical approaches used in photosynthesis studies. FCM on the other hand, and despite a promising start in the 90's (Ashcroft *et al.* 1986, Xu *et al.* 1990, Schröder and Petit, 1992, Pfundel *et al.* 1996), has seldom been applied to study the functional aspects of photosynthetic processes. FCM allows measuring the physical-chemical proprieties of particles flowing in a fluid stream, combining high speed (up to thousands of events per second), accuracy and high sensitivity (distinguishing variation in the submicron range, Dubelaar and Jonker 2000), to the capability of performing multi-parametric assays through the simultaneous analysis of fluorescence and the light scattering proprieties of particles (Loureiro *et al.* 2006).

FCM's ability to evaluate the interaction between particle and light beam, can be used to characterize the morphology and integrity of chloroplast: light reflected in small angles (Forward Scatter – FS) is related to the particle's volume/size while light reflected in large angles (Side Scatter – SS) is related to the particle's granularity/internal organization. Schröder and Petit (1992) demonstrated that a combination of FS and SS alone could be used to discriminate between intact and disrupted chloroplasts. Morovoer, a comparison between the results obtained by FCM and the ferricyanide test (a common test used to evalute chloroplast integrity) demonstrated that both techniques provided highly similar results (80% intact chloroplast by ferricyanide test, 76% by FCM). FCM presents several advantages over other methods used to evaluate chloroplast integrity (*e.g.* ferricyanide test, phase-contrast microscopy, gluconate-6-phosphate dehydrogenase activity), namely, as it can be used to evaluate thousands of chloroplasts per second with great accuracy while simultaniously analyzing other parameters, whether it be autofluorescence or the fluorescence emitted by a labelled target. Furthermore, due to the high sensitivity of FCM, subpopulations can be detected and special types of cytometers called fluorescence-activated cell sorters can be used to separate, purify and recover populations of interest for further analyses. In what concerns chloroplast autofluorescence, Xu *et al.* (1990) theorized that a 488 nm flow cytometer laser is able to detect the Chl *a* fluorescence emitted when the PSII reaction centers are locked in the Q_A^- state, that is, maximum fluorescence, opening the possibility of using this technique to study functional aspects of the photosynthetic apparatus.

The objective of the investigation presented here was to evaluate the applicability of FCM in phytotoxicity studies, by comparing FCM and PAM measurements, in order to understand if the parameters provided by FCM can be used as biomarkers of photosynthetic impairment; using Paraquat, a herbicide known to affect photosynthesis (Bromilow 2004), as a model stress factor.

Materials and methods

Plant growth and experimental conditions: *Pisum sativum* L. cv. Telephone seeds were germinated and grown in a peat/perlite mixture (4:1) for three weeks, at a temperature of 24ºC ± 1ºC, under a light intensity of 200 µmol m^{-2} s^{-1} and photoperiod of 16 h/8 h (light/dark). After this, were sprayed with a photosynthesis acting herbicide containing Paraquat as the active agent at the maximum permitted concentration for field spraying (1% w/v). Plants were analyzed at 3, 6, 9, 12, 15, 18, 21, and 24 h of exposure to the herbicide, and were adapted to the dark for 30 min prior to isolation/analysis.

Results presented are the outcome of 3 independent experiments.

Chloroplast isolation and FCM analysis: For FCM analysis, chloroplast isolation was performed using the protocol developed by Robinson and Mant (2002) with some modifications (4,000 × *g*, centrifugation times were increased from 1 to 2 min and nylon mesh was used to filter the suspension instead of miracloth). In brief, leaves were harvested as needed and ground with a mortar and pestle (automated devices can be utilized instead), in Hepes-Sorbitol buffer (HS), containing sorbitol and Hepes. The suspension was filtered through two 50 µm nylon meshes, and placed in a swing out rotor centrifuge for 2 min at 4,000 × *g*. Supernatant was collected and placed in 2 ml of a 35% Percoll® solution, and centrifuged for 8 min at 1,400 × *g*. Pellet was collected, resuspended and centrifuged twice in HS buffer at 4,000 × *g* for 2 min, to wash the Percoll® away and then analyzed in a flow cytometer (*Coulter Epics XL, Beckman Coulter*, Hialeah, FL, USA). The FS and SS of each particle was registered; the autofluorescence (as relative fluorescence intensity, FL) emitted by chloroplasts was collected using an argon ion laser operating at 488 nm, a 645 nm dichroic long pass filter and a 675 nm band pass filter. Three individuals were analyzed per condition and three replicates were sampled by individual. From the analysis of the level of FL emitted and the FS and SS values of control chloroplasts, two populations were defined, A and B. Population B comprises particles with high FL and well defined scatter values (intact chloroplast); population A includes particles with low FL emission and diffuse scatter values, lower than those of Pop B (damaged chloroplast). This classification was validated by comparing this data with the one resulting from the

analysis of chloroplasts damaged by osmotic stress (incubated 30 min in a solution containing 1% v/v Triton X–100 and 100 mM of NaCl, pH 7.5). With these parameters set, the voltage of the cytometer and the regions characterizing these populations were kept constant for the remaining of the assay. The results and images were acquired and produced using the *SYSTEM II* software version *3.0* (*Coulter Electronics*, Hialeah, FL, USA).

PAM fluorometry: Variable Chl fluorescence was measured in intact leaves, using a portable *PAM* fluorometer (*Portable Junior-PAM*, *Gademann Instruments GmbH*, Germany). This instrument applied a modulated blue light (LED-lamp peaking at 470 nm, half-bandwidth of 31 nm) as a source for measuring actinic and saturating light, emitted at a frequency of 25 Hz when measuring the minimum fluorescence level (F_0) or 1.2 kHz when measuring other fluorescence parameters. Fluorescence levels F_0 and F_m were measured by applied light intensities of < 0.1 mmol m^{-2} s^{-1} (measuring light) and > 5,000 mmol m^{-2} s^{-1} (saturating pulses). Fluorescence was measured *in situ* using a 1.5 mm-diameter plastic fibre-optics (*Edmund Optics*, UK), maintained at a constant distance of 2 mm from the leaf surface. After a 15-min period of dark adaptation, one saturating pulse of 0.8 s was applied to measure F_0 and F_m and to calculate F_v/F_m.

Pigment content: Chl *a* and Chl *b* were quantified spectrophotometrically, following the protocol developed by Sims and Gamon (2002). After grinding in an acetone:Tris (80:20, v/v) buffer solution, the suspension was vortexed and centrifuged at 2,800 × *g* for 5 min. The supernatant was stored in ice and covered with aluminium foil and the pellet was treated as above. The new supernatant was added to the previous one, and then the sample was diluted to a final volume of 6 ml. The absorbance of 4 individuals per condition (3 replicates per individual) was collected in the following wavelengths 537, 647, and 663 nm, which then were used to calculate Chl *a* and *b* concentrations with the following formulas:

$$\text{Chl } a = 0{,}01373\ A_{663} - 0{,}000897\ A_{537} - 0{,}003046\ A_{647}\ ;$$
$$\text{Chl } b = 0{,}02405\ A_{647} - 0{,}004305\ A_{537} - 0{,}005507\ A_{663}$$

Statistical analysis: Data collected were analyzed with a one-way *ANOVA*, using *SPSS 17*. Whenever significant differences ($P<0.05$) were detected, a *Holm-Sidak* multiple-comparison test was performed. Due to the similarity between the controls of each hour of exposure, for display and statistical purposes, these were considered as a single control condition for all the analyses. FCM and PAM flourometry data were correlated by *Pearson*'s correlation using *SigmaPlot for Windows 11.0*.

Results

Physical characterization: Paraquat exposure caused severe effects in the green parts of the plants, especially after 12 h of exposure (Fig. 1*C,D*), with progressive wilting, loss of brightness and graying of the leaves, culminating with the death of the aerial part of the plant in about 48 to 72 h of exposure.

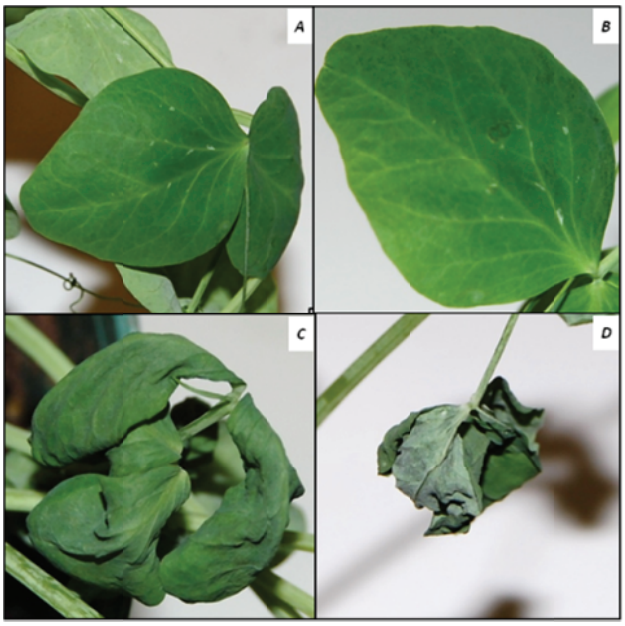

Fig. 1. Leaves of *Pisum sativum* exposed to Paraquat. *A*: control, *B*: 12 h, *C*: 15 h, and *D*: 24 h of exposure.

FCM analysis: Results from the analysis of intact and damaged chloroplast (osmotic stress) are presented in Fig. 2. The FS *vs.* SS cytogram of intact chloroplast (Fig. 2*A*) shows a well defined population of chloroplasts; on the other hand, broken chloroplasts (Fig. 2*B*) have heterogeneous distribution and lower scatter values. When comparing the aspect of intact and damaged chloroplast (Fig. 2*C* and *D*, respectively) it is easily observable that osmotic shock leads to chloroplast burst, eventually disintegrating it into smaller fragments. The overlay of intact (Fig. 2, *gray*) and broken (Fig. 2, *white*) chloroplast's FL histogram demonstrates that the main population of intact chloroplast emits higher FL than broken/damaged ones. Within the controls, a small % of the chloroplast emits lower FL, overlapping with the histogram of damaged chloroplast. This subpopulation also had lower scatter values than those of high FL emitting chloroplast, presenting an overall profile alike damaged chloroplast, and was thus defined as Pop A.
Exposure to Paraquat caused significant changes in the volume and granularity of chloroplast, when compared to controls (Fig. 3*A*). For Pop A, after some heterogeneous variation until 9 h of exposure, a steady and significant

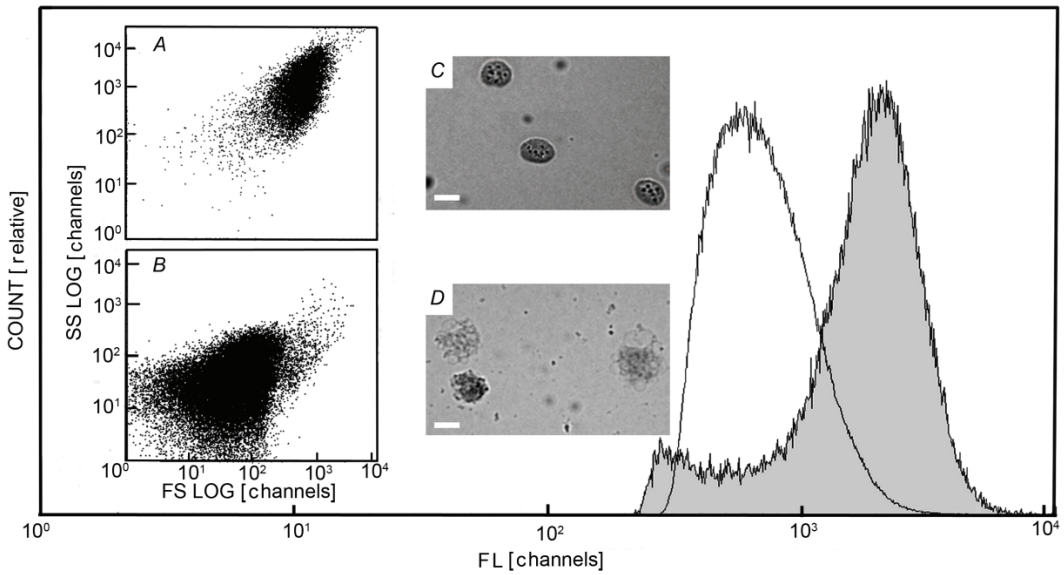

Fig. 2. Analysis of intact and broken/disrupted chloroplast. The relative fluorescence intensity (FL) histogram presents an overlay of intact (*grey*) and osmotically shocked chloroplast (*white*). Inserted in the figure are the FS vs SS cytograms of isolated (*A*) and osmotically shocked chloroplasts (*B*) as well as the pictures of those chloroplast taken with a 40 × magnification (*C*: intact, *D*: osmotically shocked).

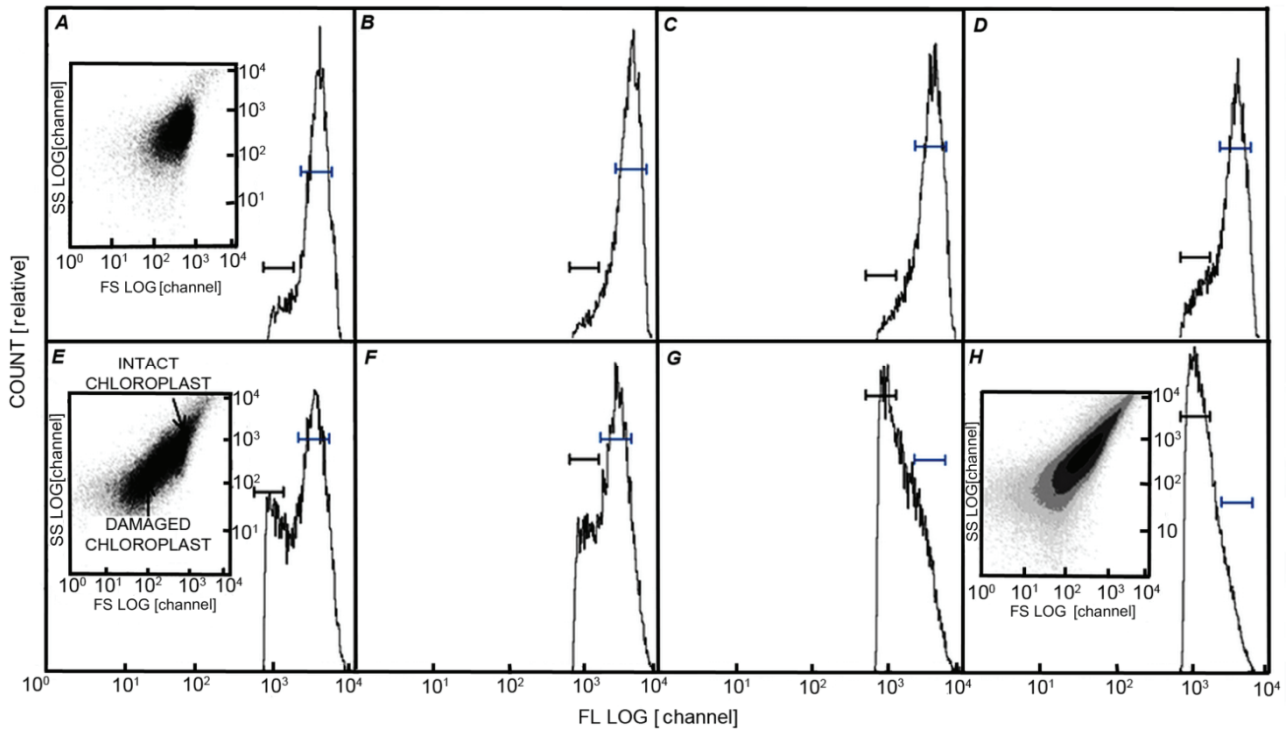

Fig. 3. FL histograms of chloroplasts isolated from plants exposed to Paraquat. *A*: control, *B*: 3 h, *C*: 6 h, *D*: 9 h, *E*: 12 h, *F*: 15 h, *G*: 18 h, and *H*: 24 h of exposure (the histogram at 21 h of exposure was omitted due to its similarity with the one at 24 h). In each histogram 2 regions are marked, the blue region defines Pop B and the black one defines Pop A. Inserted in histograms *A*, *E*, and *H* are the respective cytograms of FS *vs*. SS in logarithmic scale ($n = 9$).

increase was observed in the subsequent hours, peaking at 24 h of exposure (Fig. 4*A*).

The volume and granularity values of Pop B presented different responses during the first 3 times of analysis after exposure to Paraquat (3, 6, and 9 h); if volume decreased from 0 to 9 h of exposure (45% decrease), granularity increased from 0 to 3 h, and then decreased until 9 h of exposure (Fig. 4*B*). After 9 h of exposure,

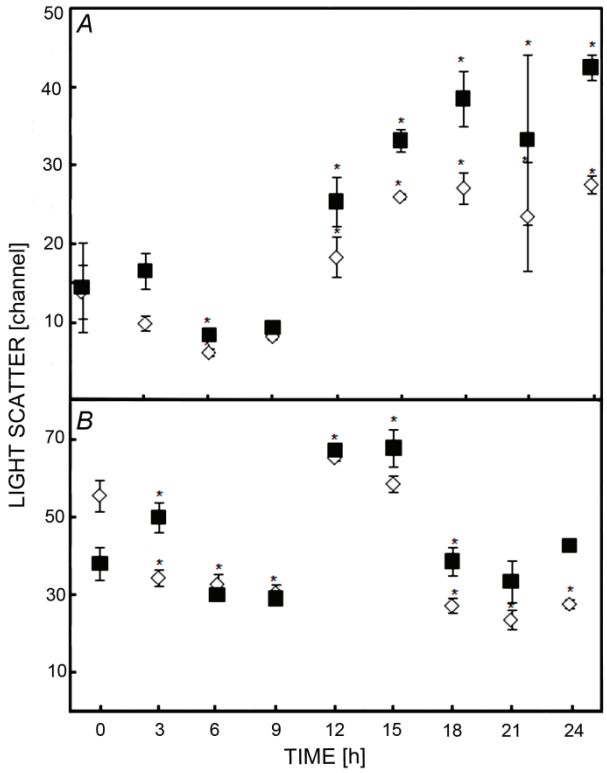

Fig. 4. Volume (FS– diamond) and granularity (SS– closed squares) variation of chloroplasts isolated from plant exposed to Paraquat for the given time. Values given are the mean and standard deviation (as error bars) of Pop A (*A*) and Pop B. (*B*). Values marked with a (*) are significantly different from control ($P \leq 0.05$) ($n = 9$).

both parameters presented the same variations: a sharp increase from 9 h to 12 h (around 55%) and then a high decrease from 15 to 21 h (60% for FS, 50% for SS, lowest values observed). Nonetheless, the decrease was more pronounced in volume, being that the last 3 analysis points (18, 21, and 24 h) were significantly lower ($P<0.05$) than the control, while for the granularity, the decrease for this time gap, leveled the values with the control ones. The maximum variation observed for FS was between 12 and 21 h (a decrease of 64%; 58% between control and 21 h) while for SS, the highest variation was observed between 9 and 15 h (increase of 58%; 44% between control and 15 h).

The mean and median FL for populations A and B were collected and compared but there were not significant differences between the mean and median FL ($P>0.05$), within any of the populations.

Pop A showed little variation in FL ($P>0.05$) throughout the experiment (Table 1, Fig. 3*F*,*G*,*H*), with the largest variation being around 10% (between 6 and 18 h of exposure).

Pop B's FL suffered significant variation as time progressed: significant increase was observed from 0 to 6 h of exposure ($P \leq 0.05$) followed by a decrease to control levels at 9 h of exposure ($P>0.05$). From 12 h of exposure onwards, FL values are significantly lower than control ($P \leq 0.05$), with a sharp decrease between 12 and 15 h, followed by a less pronounced decrease until 21 h, where the lowest value was observed (40% lower than control).

The % of intact chloroplasts (Pop B) suffered little variation within the first 12 h of exposure to Paraquat ($P>0.05$). After this, a constant decrease in the percentage of intact chloroplast was observed, being the lowest value (19%) observed at 24 h of exposure (Table 2). The % of chloroplasts in Pop A increased with time of exposure reaching a maximum of 81% at 24 h after exposure.

Table 1. Fluorescence emission of chloroplast extracted from plants exposed to Paraquat for the given time. Parameters presented are: fluorescence intensity (FL), of intact chloroplast (Pop B); broken/damaged chloroplast (Pop A) and both populations [Pop (A+B)]. Values are given as mean/median ± SD for each population. Values followed by *different letters* are statistically different ($P \leq 0.05$).

T [h]	FL log [Channels]					
	Pop B		Pop A		Pop (A+B)	
	Median	Mean	Median	Mean	Median	Mean
0	361.4 ± 8.5^{a}	364.3 ± 11.9^{a}	98.8 ± 1.0^{a}	97.3 ± 1.2^{a}	300.1 ± 11.7^{a}	340.5 ± 15.3^{a}
3	406.4 ± 9.4^{b}	385.2 ± 1.2^{b}	101.6 ± 2.6^{a}	100.2 ± 1.1^{a}	265.1 ± 7.9^{a}	313.9 ± 2.0^{a}
6	458.5 ± 26.6^{b}	428.3 ± 55.7^{b}	107.0 ± 4.5^{a}	105.8 ± 4.1^{a}	209.4 ± 28.1^{b}	202.4 ± 40.5^{b}
9	333.5 ± 7.7^{ac}	314.0 ± 1.2^{ac}	101.0 ± 1.4^{a}	99.2 ± 2.0^{a}	258.0 ± 3.9^{ab}	309.4 ± 5.3^{ab}
12	322.1 ± 15.4^{c}	316.9 ± 7.6^{c}	100.4 ± 4.1^{a}	98.9 ± 3.6^{a}	233.6 ± 2.4^{b}	258.8 ± 3.9^{b}
15	278.8 ± 3.6^{d}	281.5 ± 1.1^{d}	100.8 ± 2.7^{a}	99.6 ± 1.1^{a}	218.8 ± 1.4^{b}	234.3 ± 1.3^{b}
18	238.2 ± 5.4^{d}	249.6 ± 1.0^{d}	95.7 ± 3.7^{a}	93.3 ± 4.6^{a}	166.6 ± 2.9^{c}	153.0 ± 1.0^{c}
21	200.5 ± 1.9^{e}	211.1 ± 7.4^{e}	99.6 ± 5.5^{a}	99.7 ± 10.0^{a}	147.8 ± 3.6^{c}	135.4 ± 1.1^{c}
24	208.2 ± 1.3^{e}	224.3 ± 1.1^{e}	105.0 ± 4.8^{a}	103.2 ± 4.0^{a}	149.6 ± 16.1^{c}	142.4 ± 6.4^{c}

Photosynthetic yield analysis: The variation of F_v/F_m, F_m and F_0 along the time of exposure is presented in Fig. 5. F_v/F_m decreased markedly throughout the experiment; the values measured after the first 12 h of exposure were significantly higher than those recorded during the last 12 h. The lowest set of values were observed after 24 h of exposure to Paraquat, which were significantly lower ($P<0.05$) than all other values. The F_0 presented little variation throughout the duration of the experiment ($P>0.05$), being that the maximum variation, in respect to

Table 2. Percentage of intact (Pop B) and damaged/broken chloroplast (Pop A) along time of exposure to Paraquat. Values are given as mean percentage ± SD of the mean. In each column, values followed by *the same letter* are not statistically significant (P>0.05), (n = 9).

Exposure time [h]	Pop B [%]	Pop A [%]
0	75 ± 6.4 ^{ab}	25 ± 2.1^{ab}
3	78 ± 5.6^{ab}	22 ± 6.2^{ab}
6	83 ± 5.7^{ab}	17 ± 10.1^{ab}
9	87 ± 6.7^{a}	13 ± 3.2^{a}
12	64 ± 4.5^{b}	36 ± 6.9^{b}
15	77 ± 5.5^{ab}	23 ± 4.1^{ab}
18	32 ± 3.2^{c}	68 ± 9.0^{c}
21	28 ± 2.8^{c}	72 ± 7.2^{c}
24	19 ± 1.8^{c}	81 ± 6.8^{c}

control, was of 23% (12 h exposure). Regarding F_m, after 3 h of exposure this parameter had already suffered a significant plunge of 40% in respect to the control (P≤0.05). After a slight increase at 6 h, F_m steadily decreased until 15 h of exposure (74% lower than at 0 h) and then little variation (P>0.05) was seen until 24 h of exposure (77% lower than control).

Correlation between FCM and PAM parameters: The relationship among the endpoints evaluated by FCM and PAM fluorometry was analyzed using a *Pearson*'s correlation (Fig. 6, Table 3). Also and in order to better correlate FCM data with PAM fluorometry, in addition to Pop A and Pop B, a third population was also considered for analysis, consisting in a combination of Pop A and Pop B [Pop (A+B)]. The FL of Pop B (mean or median) and the F_v/F_m presented the best correlation of all the parameters assayed (r = 0.883 and P=0.0016), followed by the FL of Pop (A+B) and the F_v/F_m (mean FL r = 0.810, P=0.004; median FL r = 0.842, P=0.008). The F_m (P≤0.02) also had a positive correlation with the FL of Pop B and Pop (A+B) but to an inferior degree of that observed for the F_v/F_m. The F_0 did not present significant correlation with any of the parameters assayed; in fact, the correlation coefficients were all negative. Likewise, Pop A did not present significant relationship with F_v/F_m, F_0, or F_m (P>0.05).

Pigment quantification: Pigment content was the only parameter that was not affected by Paraquat exposure as the quantification of both types of Chl presented no significant variation (P>0.05) throughout the experiment (Table 4).

Discussion

The characterization of the chloroplast population allowed distinguishing between intact chloroplasts (well defined scatter values and high FL) and broken/damaged chloroplast (low FL and scatter values). The classification of the events in Pop A, as broken/damaged chloroplast is supported by the analysis done to osmotically broken chloroplasts, which presented similar profiles to those of Pop A. These observations are in agreement with the previous FCM reports (Ashcroft *et al.* 1986, Schröder and Petit, 1992), in which populations were defined based on fluorescence emission and scatter values. From this, we can conclude that the methodology adopted to isolate chloroplast used in our investigation provides a good yield of intact chloroplasts (nearly 75%) and is suitable for FCM studies.

The biomarkers assessed by FCM were severely affected by Paraquat being that the ones related to morphology were more sensitive than FL. This statement is based on the fact that significant structural modifications were observed prior to any significant variation in FL. After 3 h of exposure, intact chloroplasts decreased in volume but their internal complexity and FL

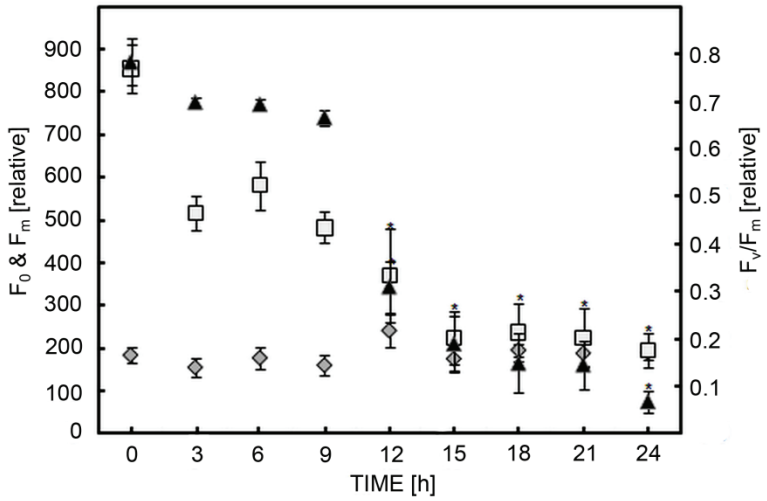

Fig. 5. Variation in F_v/F_m (right axis, *closed triangle*), F_0 (*diamond*) and F_m (left axis, *open square*) of pea plants after exposure to Paraquat. Values are presented as mean plus error bar (SD of the mean) (n = 10).

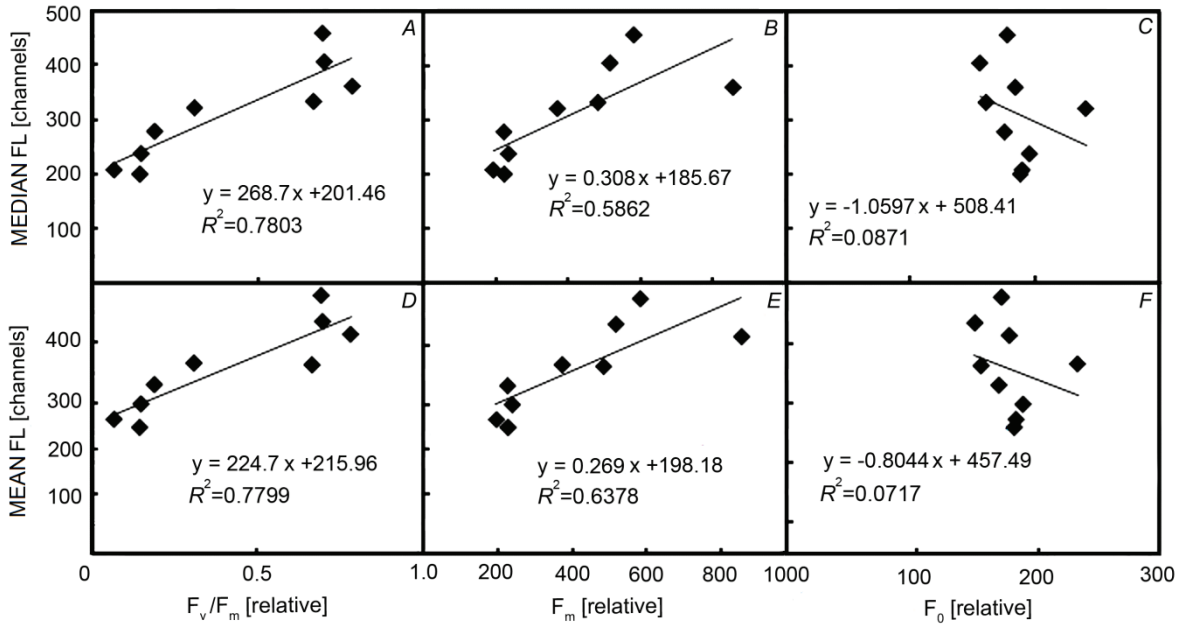

Fig. 6. Correlation graphics between FL and F_v/F_m, F_m or F_0, for Pop B. The graphics are relative to the median (*A*: F_v/F_m; *B*: F_m and *C*: F_0) and mean (*D*: F_v/F_m; *E*: F_m and *F*: F_0) FL value. Inserted in each graph is given the equation for the linear regression fitted to the data. The data obtained by PAM fluorometry were measured directly from intact leaves while FCM's data were collected from isolated chloroplasts.

Table 3. Pearson's correlation coefficient among the FL (mean or median) of the populations evaluated (A, B and A+B) and the F_0, F_m, and F_v/F_m of plants exposed to Paraquat. Level of significance is represented by *($P \leq 0.05$), ** ($P \leq 0.001$) or n.s. ($P > 0.05$). The F_0, F_m, and F_v/F_m were measured in intact leaves while the FL was measured in extracted chloroplasts.

		F_0	F_m	F_v/F_m
FL Pop A	Mean	$-0.202^{n.s.}$	$0.067^{n.s.}$	$0.177^{n.s.}$
	Median	$-0.205^{n.s.}$	$0.081^{n.s.}$	$0.196^{n.s}$
FL Pop B	Mean	$-0.268^{n.s.}$	0.799^{**}	0.883^{**}
	Median	$-0.295^{n.s.}$	0.766^{*}	0.883^{**}
FL Pop (A+B)	Mean	$-0.274^{n.s.}$	0.767^{*}	0.842^{**}
	Median	$-0.244^{n.s.}$	0.830^{**}	0.810^{**}

Table 4. Pigment concentration (Chl *a* and Chl *b*) extracted from pea plants exposed to Paraquat for the given time. Values are presented as mean ± SD of the mean, ($n = 12$).

Time [h]	Chl *a* [μmol ml^{-1}]	Chl *b* [μmol ml^{-1}]
0	0.226 ± 0.0369	0.111 ± 0.0200
3	0.258 ± 0.0238	0.113 ± 0.0081
6	0.259 ± 0.0248	0.131 ± 0.0096
9	0.267 ± 0.0409	0.132 ± 0.0142
12	0.273 ± 0.0148	0.123 ± 0.0156
15	0.211 ± 0.0292	0.093 ± 0.0162
18	0.221 ± 0.0430	0.100 ± 0.0215
21	0.262 ± 0.0413	0.111 ± 0.0064
24	0.242 ± 0.0343	0.120 ± 0.0193

significantly surpassed the ones of the control. This might be due to a rearrangement of the chloroplast's grana all together with a stimulation of the electron transporter chain of the PSII, resulting in the observed FL increase. This hypothesis is supported by the reports of Po and Ho (1997) and Neuhaus and Stitt, (1989) in which small doses of Paraquat increased electron transport due to its action as a catalytic electron acceptor at the acceptor side of PSI, allowing a net transfer of electrons from water through PSII and PSI. In our case and despite the fact that a high dose was used, it is likely that the response of chloroplast to the first hours of exposure could have mimicked the effect of lower dosage and as time progressed, the effects evolved to what those authors observed for higher doses (inhibition of the process).

The progressive decrease in Pop B's FL (shift to the low FL zone) observed in Fig. 3 is in agreement with the work of Schröder and Petit (1992) that detected the same shift in the FL peak of osmotically shocked chloroplasts. We also found a high similarity between the profiles of chloroplast extracted from Paraquat-treated plants (Fig. 3) and osmotically lysed ones (Fig. 2*B*) suggesting that Paraquat induces osmotic-like stress. Data provided by Iturbe-Ormaetxe *et al.* (1998) supports our findings; they demonstrated that water deficit and Paraquat exposure produced similar symptoms in pea plants. Moreover, the sudden and high increase followed by the rapid drop in volume observed between 9 and 18 h of exposure is consistent with a swelling of the chloroplast, followed by their burst. This, together with the substantial decrease of FL, indicates that the critical period of Paraquat exposure is situated between 9 and 18 h after exposure and that it is

highly likely that the osmotic-like stress induced by Paraquat is responsible for the decrease in FL observed.

Leaves from control plants presented values of F_v/F_m around 0.8, which is close to the maximum values obtained for healthy plants (Krause and Weis 1991, Maxwell and Johnson 2000). Paraquat exposure caused a constant decrease in F_v/F_m, but this was only significant after 12 h of exposure (decrease of 50% to control) being in agreement with the observed pattern of FL decrease in FCM. Nonetheless, FCM seems to be more sensitive to small variations in fluorescence emission, detecting subtle changes that were not detected by PAM fluorimetry.

Previous studies trying to elucidate what were the characteristics of FL as measured by FCM, lead to contradictory conclusions: Ashcroft *et al.* (1986) concluded that FCM measured F_0 while Xu *et al.* (1990) found evidence that FCM measured F_m. The latter demonstrated that increasing the incubation temperature caused a decrease in fluorescence intensity (like what was expected for F_m but not for F_0) and that the application of Diuron (herbicide that inhibits photosynthetic electron transport by occupying the binding site of QB) caused little variation in fluorescence intensity (once again more like F_m than F_0). They also argued that due to the high intensity of the cytometer's light source, all the reaction centers underwent at least one turnover and the system reached the F_m state. Our results showed that FL (mean or median) correlated better with F_v/F_m than with F_m and not at all with F_0. It is worth mentioning that in the work of Xu *et al.* (1990) all the experiments were carried out in order to establish if FL presented was more similar to F_0 or F_m; the F_v/F_m was not considered. In agreement with our findings, Neale *et al.* (1989) working with in *Chroomonas* sp. found that, despite the high light intensity of a cytometer (3×10^5 times higher than full sunlight), the short interval of exposure was not enough to allow the detection of F_m and it was more likely that FCM provided values that were between F_0 and F_m.

Finally, our data also showed that F_v/F_m correlated better with the FL of intact chloroplasts than with Pop (A+B) or damaged chloroplasts (Pop A). These correlations are especially meaningful considering that, despite the data obtained through PAM fluorometry measured on leaves containing chloroplasts in various physiological and structural states, they correlate better with the FL values provided by the intact chloroplasts population than with the FL of Pop (A+B) (all the chloroplasts).

The quantification of Chl *a* and *b* did not reveal significant effects caused by Paraquat and together with the results obtained with FCM and PAM we can state that the reduction in fluorescence parameters is due to the loss of chloroplasts integrity rather than by a decrease in Chl content. Reports like Franqueira *et al.* (1999), Ekmekci and Terzioglu (2005) might explain why we did not find significant variation of pigment content as a result of Paraquat exposure. Despite Franqueira *et al.* (1999) having found some variation within the first 24 h after exposure; only at 96 h were the effects significant. In the work of Ekmekci and Terzioglu (2005), pigment quantification was only performed after 48 h of exposure (all the other parameters were analyzed within the first 24 h) and in new leaves rather than developed ones, which might be consistent with the fact that Paraquat-induced effects in pigment content are only truly significant after 24 h. However other authors (Mascher *et al.* 2005, Iturbe-Ormaetxe *et al.* 1998) have detected a significant decrease in pigment content within the first 8 h after Paraquat exposure. Unlike in our work, however, those authors samples were exposed to a much higher light intensity than the one used by us and Paraquat's effects require and are intensified by high light intensities (Varadi *et al.* 2000). Considering the variations in volume detected by FCM (indicative of chloroplast burst) it is possible that in our work, chloroplast destruction occurred prior to degradation of chlorophyll.

The data presented here have demonstrated that the most sensitive endpoint to Paraquat exposure was the chloroplast's structure (both FS and SS) which showed significant differences at the first point of analysis (3 h); variation in fluorescence emission (either FCM or PAM) was only truly meaningful after 12 h of exposure. These results indicate that Paraquat causes a disruption of the chloroplast's integrity which provokes a gradual loss of function of the electron transport chain.

We also demonstrate that FL correlated significantly with F_v/F_m and better than with F_m or F_0; this information is an important contribution that helps to clarify contradictory reports claiming that FL had characteristics of F_0 or F_m.

In conclusion, FCM proved to be an effective and robust tool for assessing chloroplast functionality and it is expected that this investigation encourages researchers to explore the potentialities of this technique and provide new insights in photosynthesis.

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