CHLOROPHYLL FLUORESCENCE OF BARLEY (*H. VULGARE* L.) SEEDLINGS GROWN IN EXCESS OF CD*

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Summary. The effect of excess of cadmium (Cd) on chlorophyll fluorescence of barley seedlings was studied. The leaves were dark-adapted and then illuminated at 10 different light intensities within the range of 70–1600 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation (PAR). It was found that Cd decreases the chlorophyll fluorescence parameters in dark-adapted leaves: ground (F_o), maximal (F_m) and variable (F_v) fluorescence. However the F_v/F_m ratio was slightly influenced. At light intensities below $400\,\mu mol.m^{-2}.s^{-1}$ PAR no significant differences between values of photochemical quenching (qP), non-photochemical quenching (qN) and yield of photochemical energy conversion (Y) of control and Cd-treated plants were found. At light intensities about $500\,\mu mol.m^{-2}.s^{-1}$ and higher significant decrease in qP, concomitant increase in qN as well as a reduction in Y were observed. In Cd-treated plants the maximum of apparent electron transport rate (ETR) were reached earlier than in control plants and under lower PAR intensities, indicating that the energy consumption by Calvin's cycle reactions is reduced. It is speculated that excess of Cd in barley plants causes a down regulation of PS II to avoid over-reduction of primary electron acceptor Q_A and to reduce the load on the electron transport chain.

Key words: barley, cadmium, chlorophyll fluorescence

Abbreviations: F_o, F_v and F_m – ground, variable and maximal fluorescence of dark-adapted samples; F and F'_m – ground and maximal fluorescence of light-adapted samples; qP – photochemical quenching; qN – non-photo-

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chemical quenching; Y – yield of photochemical energy conversion; ETR – apparent electron transport rate, PAR – photosynthetically active radiation; SLP – saturating light pulse

Introduction

The negative effect of Cd on photosynthesis is known for a long time, but has not been fully understood till present. Despite of well-established negative effects of Cd on chlorophyll content (Stobart et al., 1985; Somashekaraiah et al., 1992), chloroplast ultrastructure (Barcelo et al., 1988; Stoyanova and Chakalova, 1990), enzyme activities (Stiborova et al., 1986; Chugh and Sawhney, 1999), photochemical processes (Baszynski et al., 1980; Siedleska and Krupa, 1996) etc, a clear picture of overall photosynthesis response to excess of Cd is absent.

There are different opinions about the primary site of the toxic Cd effect on photosynthesis. Some authors have emphasized on direct effect of Cd on the membrane-bound photochemical reactions (Baszynski et al., 1980; Becerril et al., 1989), while others have concentrated on some Calvin's cycle enzymes (Stiborova et al., 1986; Sheoran et al., 1990). One of the possible reasons for this disagreement is the fact that results obtained by destructive analyses do not always represent the real situation *in vivo*. For example, the data about high susceptibility of PS II to Cd stress analysed *in vitro* are not always confirmed when its photochemical activity is evaluated by chlorophyll fluorescence in dark-adapted leaves (Greger and Ogren, 1991; Krupa et al., 1992).

By use of a fluorescence quenching analysis during steady-state photosynthesis Krupa et al. (1993) have established that Cd reduced photochemical quenching (qP) and stimulated non-photochemical quenching (qN) in been leaves. The increase of qN they explained as a consequence of disturbed utilization of photosynthetic energy. As its major sink are the Calvin's cycle enzyme reactions, the authors supposed that the inhibition of enzyme reactions results in an excess of reducing equivalents and feedback inhibition of PS II. The decrease of qP is discussed as a mechanism to avoid over-reduction of the primary electron acceptor of PSII – Q_A. The above hypothesis was confirmed by Siedlecka et al. (1997), who established that the adenilate pool in the leaves of Cd-treated bean plants is increased.

In our previous investigations with Cd-treated barley plants we found that the rate of CO_2-fixation decreased by about 20% which was accompanied with disorders in chloroplast ultrastructure, lower rate of ^{14}C-incorporation in primary photoproducts, lower content of photosynthetic pigments, etc. (Vassilev et al., 1995, 1997, 1998). At the same time the functional activity of PSII according to fluorescence analysis in dark-adapted leaves was only slightly reduced. In order to clarify the mechanism of action of Cd on PS II photochemistry *in vivo* we have followed the changes of the

main chlorophyll fluorescence parameters of control and Cd-treated plants both in dark- and light-adapted leaves.

Materials and Methods

Barley plants (*Hordeum vulgare* L., cv. Hemus) were grown as water culture as described elsewhere (Vassilev et al., 1997). One part of them was subjected to a long-term Cd stress – 12 days with 54 µM Cd. At the end of this period the chlorophyll fluorescence of the control and Cd-treated plants was determined on the first whole-expanded leaf by a pulse modulation fluorometer (MINI-PAM, Heinz Walz, Germany).

The measurements were made on the top region of the selected leaves. Because of expressed heterogeneity of Cd-toxicity the measurements were made on the same region of different leaves – without and with initial signs of leaf necrosis (mentioned as non-necrotic and necrotic). After at least 30 min in darkness the values of the ground (F_o) and maximal fluorescence (F_m) were recorded at measuring light 0.15 µmol.m^{-2}.s^{-1} and saturating light pulse (SLP) – 5000 µmol.m^{-2}.s^{-1}, respectively, for 0.8 s.

After that plants were illuminated for 10–15 min with ambient light close to this in the growing room (approx. 70 µmol.m^{-2}.s^{-1} PAR) till reaching steady-state photosynthesis and SLP was applied. Than the internal source of actinic light was switched on and plant samples were illuminated with 9 levels of PAR intensity – about 90, 120 150, 190, 250, 390, 570, 1000 and 1600 µmol.m^{-2}.s^{-1}. The length of each illumination period before SLP to be applied was 2 min, which was recommended for avoiding excessive internal heating of the MINI-PAM (Handbook of operation with MINI-PAM, 1996). During this period the leaves reached near steady-state photosynthesis at the new light conditions as judged by the stabilization of F reading on the display.

At every level of PAR intensity the following fluorescence parameters were recorded:

F – present fluorescence of plant sample before saturation pulse;

F'_m – maximal fluorescence of the illuminated plant sample;

$Y = (F'_m - F) / F'_m$ – actual yield of photochemical energy conversion (Genty et al., 1989);

$ETR = Y \times PAR \times 0.5 \times 0.84$ – apparent electron transport rate (Handbook of operation with MINI-PAM, 1996). The computer program Table Curve for Windows 1.10 was used to estimate ETR = f(PAR).

$qP = (F'_m - F) / (F'_m - F_o)$ – photochemical quenching;

$qN = (F_m - F'_m) / (F_m - F_o)$ – non-photochemical quenching.

It is known that at high levels of qN (exceeding approx. 0.4) there can be significant quenching of F_o, resulting in a lowered yield F'_o (Bilger and Schreiber, 1986; Handbook of operation with MINI-PAM, 1996). However the MINI-PAM does not

feature an intrinsic far-red light source, necessary for correct measuring of F_o'. In order to avoid incorrect values of qP and qN we restricted our observation on this parameters to moderate light intensities, when qN did not exceed 0.4–0.5 (see Table 2).

Results

Data in Table 1 show that after Cd-treatment all fluorescence parameters of dark adapted leaves decrease considerably. This is gradually expressed from non-necrotic leaves to the leaves with initial signs of necrosis. However the decrease of the different parameters is not of the same extent. Compared to F_m the changes of F_o are relatively slight – only by about 7% at non-necrotic leaves, while F_m decreases by 25% at the same sample. The corresponding decreases of F_o and F_m in the necrotic leaves are by 25 and 38%. Because of this specific changes of F_o and F_m values the parameter $F_v = F_m$ - F_o was greatly reduced at non-necrotic leaves by about 29% and 42% at necrotic leaves.

More reduced values of F_v lead to decrease in F_v/F_m ratio. A decline of this ratio by about 6% (control – 0.79 and Cd-treated leaves – 0.74) is significant in both non-necrotic and necrotic leaf areas. Furthermore observed values are near to the accepted norm for healthy, non-stressed leaves – 0.75–85 (Bolhar-Nordenkampf and Oquist, 1993) but still out of them.

In Table 2 the data about photochemical (qP) and non-photochemical (qN) quenching and actual yield (Y) of photochemical energy conversion are presented (Genty et al., 1989), characterizing PS II photochemistry in control and Cd-treated plants under selected light intensities. No significant differences between control and Cd-treated plants were found at low light intensities in the three examined parameters. At moderate light intensities a decrease

Table 1. Fluorescence parameters of dark-adapted leaves of control and Cd-treated plants.

Parameters	Control		Cd non-necrotic		Cd necrotic	
	Value	%	Value	%	Value	%
F_o	413.3 ± 9.5	100.0	383.4 ± 7.6	92.8[a]	309.7 ± 38.1	74.9[c]
F_m	1926.3 ±72.8	100.0	1462.3 ±60.1	75.9[c]	1190.3 ± 115.5	61.8[c]
$F_v = F_m$ - F_o	1513.0 ±67.8	100.0	1079.0 ±53.2	71.1[c]	880.3 ± 84.8	58.2[c]
F_v/F_m	0.785± 0.008	100.0	0.738± 0.006	94.0[c]	0.739± 0.003	94.1[c]

[a] at $P_{0.05}$, [b] at $P_{0.01}$, [c] at $P_{0.001}$

Table 2. Values of qP, qN and Y of control and Cd-treated plants at selected PAR intensities.

Treatment	PAR μmol.m^{-2}.s^{-1}	qP	qN	Y (Yield)
Control	120	0.918	0.285	0.663
Cd non-necrotic	”	0.945	0.273	0.663
Cd necrotic	”	0.959	0.264	0.653
Control	390	0.841	0.383	0.582
Cd non-necrotic	”	0.815	0.398	0.552
Cd necrotic	”	0.793	0.424	0.516
Control	570	0.814	0.452	0.551
Cd non-necrotic	”	0.728[b]	0.473[b]	0.500
Cd necrotic	”	0.715[b]	0.544[b]	0.441[a]
Control	1000			0.446
Cd non-necrotic	”			0.399[b]
Cd necrotic	”			0.316[b]
Control	1600			0.349
Cd non-necrotic	”			0.294[c]
Cd necrotic	”			0.217[c]

[a] at $P_{0.05}$, [b] at $P_{0.01}$, [c] at $P_{0.001}$

in qP, concomitant increase in qN as well as a reduced Y were observed (as a tendency at 390 μmol.m^{-2}.s^{-1} and significant at 570 μmol.m^{-2}.s^{-1} PAR). At higher light intensities (1000–1600 μmol.m^{-2}.s^{-1}) the decrease of Y in Cd-treated leaves is more expressed. Obviously at these conditions Cd provokes losses in the photochemical efficiency of PS II.

In order to explain this effect of Cd we analysed the dependence of apparent electron transport rate (ETR) on light intensity – ETR=f(PAR). We found that the exponential equation: ETR=a[(1 - exp(-x/c)], where a is the maximal ETR value under saturating light intensity of PAR, x is PAR intensity and c is rate constant, best describes ETR dependence on PAR.

Light response curves of apparent electron transport rate (ETR) in control and Cd-treated plants are presented in Fig. 1. They show that both PAR required for light saturation of ETR and the maximal values of ETR at light saturation decrease in the order: control, Cd-treated non-necrotic leaves and Cd-treated necrotic leaves. In Table 3 are shown calculated maximal values of ETR (parameters a) and the rate constants (parameter c), describing the light response curves. In Cd treated plants the rate constants (parameter c) are lower. It is also seen that the maximal ETR (parameter a) of control plants is approximately twice as high as in areas with initial necrosis of Cd-treated plants.
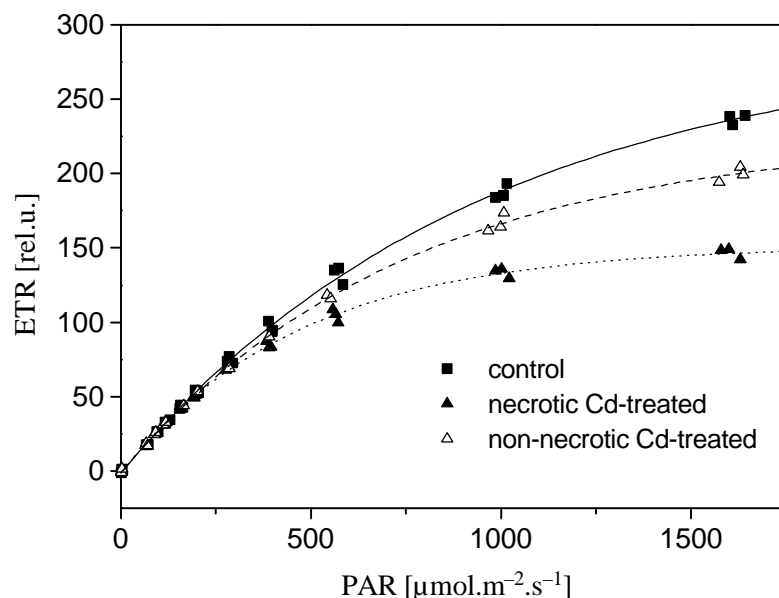
Fig. 1. Photosynthetic electron transport rate in the top region of control and necrotic and non-necrotic Cd-treated leaves

Table 3. Values of the parameters *a* (maximal ETR in the area of light saturation and *c* (rate constant) of the exponential curves, describing ETR = f(PAR)

Treatment	Parameter	Value	Std. err.	95% Confidence	
				lower limit	upper limit
Control	*a*	290.31	5.39	279.24	301.27
Cd non-necrotic	”	225.95	3.17	219.31	232.59
Cd necrotic	”	151.29	1.53	148.16	154.43
Control	*c*	953.94	34.28	883.83	1024.06
Cd non-necrotic	”	748.69	24.58	697.21	800.18
Cd necrotic	”	471.73	12.52	446.12	497.33

Discussion

As known the parameter F_o represents the emission of excited antenna chlorophyll a molecules before the excitation migrates to the reaction centres and it is independent of photochemical events (Krause and Weis, 1991). Relatively slight changes in F_o at non-necrotic leaves (Table 1) indicate that earlier effects of Cd-treatments are not directly related to the energy transfer from antenna pigments to the reaction centres. Stronger reduced values of F_o were observed when the first signs of Cd-toxicity are

evident (i. e. at necrotic leaves). Probably that is due to some degradation of chlorophyll *a* after Cd-treatment (Vasilev et al., 1998).

The parameters maximal fluorescence (F_m), variable fluorescence ($F_v = F_m - F_o$) and F_v/F_m ratio characterize the functional state of PS II in dark-adapted leaves. The F_v/F_m ratio is considered to be a measure of PS II effectiveness in the primary photochemical reactions (Buttler, 1977). The obtained data about a slight decrease of PS II effectiveness at Cd-treated barley plants (Table 1) confirmed our previously results (Vassilev et al., 1995). It should be noticed however that the slight change of F_v/F_m ratio is recorded together with a much stronger decrease of F_m and F_v values. This could be partly explained by Cd-induced disorders in chloroplast ultrastructure (Vassilev et al., 1995). In the swollen and highly disorganized thylakoids the architecture of photosynthetic units and membrane-bound electron transport processes could be disturbed. There is evidences that leaf structural status may also affect the primary fluorescence parameters even when the F_v/F_m ratio shows little or no change (Araus and Hogan, 1994).

When a leaf is illuminated its fluorescence can vary between two extreme values – F_o and F_m, which can be assessed after dark adaptation. Any fluorescence lowering may be caused either by enhanced photochemical energy conversion or by increased heat-dissipation. The quenching analysis allows to distinguish this two fundamentally different pathways of absorbed light energy conversion. qP quenching reflects the fractions of open PS II reactions centres and denotes the proportion of excitation energy trapped by them (Krause and Weis, 1991).

qN quenching is a result of various processes that are responsible for thermal dissipation of excess energy in the photochemical apparatus – high-energy state, state transitions and photoinhibition (Krause and Weis, 1991). It has an important function in the regulation of dissipation/utilization of excitation energy (Horton and Hague, 1988). The increase of qN up to 0.4–0.5 reflects mainly increased thermal dissipation at the pigment level due to acidification of the intrathylakoid lumen resulting from membrane energization (Hetherington et al., 1998).

Evidence from a number of research groups has shown that the overall quantum yield (Y) of photochemical energy conversion can be assessed by the simple expression: $Y = (F_m' - F)/F_m'$, introduced by Genty et al. (1989). Thus Y is a measure of the actual photochemical efficiency of PS II in illuminated leaves. This parameter is very reliable, as it does not depend on F_o quenching and it can be determined at high light intensities (Table 2).

Our data presented in Table 2 show that during light adaptation even at low PAR intensities the actual quantum yield decreases compared to the values of dark-adapted leaves (Table 2). That is mainly due to the non-photochemical conversion of absorbed light energy (qN) for thylakoid membrane energization. At this light conditions nearly all reaction centres of PSII in control and Cd-treated leaves remain oxidized (opened), as indicated by qP values.

At higher light intensities (390 and 570 μmol.m^{-2}.s^{-1} PAR) further decrease of Y occurs and the difference between control and Cd-treated plants is well expressed. The data of qP indicate an increased number of reduced (closed) reaction centres of PS II at Cd-treated plants (Table 2). A higher fraction of closed reaction centres denotes that light absorption exceeds the capacity of electron transport and CO_2 fixation. As a result the part of non-photochemical energy conversion (qN) increases. This is interpreted as a mechanism to reduce the "load" on the electron transport chain (Schreiber et al., 1998). In Cd-treated plants this mechanism is more pronounced under conditions of high light intensities (1000 and 1600 μmol.m^{-2}.s^{-1} PAR). On the base of Y values (Table 2) one may suppose that in such a case further decrease of qP and increase of qN at Cd-treated plants also occurs.

Since the actual efficiency of PS II (Y) is a measure of electron yield per photon, the rate of electron transport can be calculated by multiplying the efficiency by PAR (Hetherington et al., 1998). This value is divided by two to take into account that two photons are required to generate one electron and assuming that absorbed photon energy is divided equally between the two photosystems (Schreiber et al., 1998), and finally multiplying by 0.84 – the standard factor for MINI-PAM's fiberoptics (Handbook of operation with MINI-PAM, 1996). ETR can be converted to rate of oxygen evolution by dividing by four, because four electrons are generated for each molecule of oxygen released, and is linearly related in its light response to rate of gross CO_2 fixation (Hetherington et al., 1998). Bearing in mind the above-mentioned we consider our ETR results obtained using the chlorophyll fluorescence technique to correspond to the light dependent rate of CO_2 fixation at control and Cd-treated barley plants. In this context the presented in Fig. 1 data indicate that very little or no difference in ETR values (i. e. at rates of CO_2 fixation) occurs at light intensities below 200 μmol.m^{-2}.s^{-1} PAR. The ETR decrease in Cd-treated plants (especially in leaves with signs of necrosis) is more pronounced at saturating light (Table 3).

When ETR does not limit photosynthesis, the steady state is determined by the biochemical reactions of Calvin's cycle. According to Krupa et al. (1993) in Cd-treated bean plants energy consumption by Calvin's cycle reactions is reduced, reflecting in down regulation of PS II. In our previous investigations lower potential capacity for ^{14}C-assimilation in Cd-treated barley plants was found (Vassilev et al., 1997). The above-mentioned leads us to suppose that excess of Cd in barley plants causes a down regulation of PS II in order to avoid over-reduction of primary electron acceptor Q_A and to reduce the load on the electron transport chain.

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