The effect of inhibitors on photosynthetic electron transport chain in canola leaf discs

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ABSTRACT. The mechanisms of photosynthetic electron transport can be elucidated by inhibition of electron flow through the use of specific substances that, when combined with the chlorophyll chlorophyll *a* fluorescence emission was measured to investigate the effect of several inhibitors of the photosynthetic electron transport chain in canola leaf discs. Leaf discs were incubated in the dark for 2 hours in different solutions: (a) water – control; (b) DCMU at 500 μM; (c) phenanthroline at 10 mM; and (d) hydroxylamine at 10, 50, 100 and 200 mM. Similar effects were observed between DCMU and phenanthroline, the initial fluorescence value was altered, but not the maximum fluorescence, with the disappearance of the IP phase. Hydroxylamine interacted and inhibited the oxygen evolving complex and caused an imbalance between the rate of Q_A reduction by photosystem II and the rate of Q_A oxidation by photosystem I.

Keywords: DCMU, hydroxylamine, phenanthroline, oxygen evolving complex, chlorophyll fluorescence

Efeito de inibidores na cadeia de transporte de elétrons fotossintética em discos foliares de canola

RESUMO. Os mecanismos de transporte de elétrons podem ser elucidados pela inibição do fluxo de elétrons pelo uso de substâncias específicas que, juntamente com a técnica de fluorescência da clorofila, torna-se uma ferramenta importante para detalhar a cadeia de transporte de elétrons. Neste trabalho, a emissão da fluorescência da clorofila foi mensurada para investigar o efeito dos diferentes inibidores da cadeia de transporte de elétrons fotossintéticos de discos foliares de canola. Os discos foliares foram incubados no escuro durante 02 h em diferentes soluções: (a) água - controle, (b) 500 uM de DCMU, (c) 10 mM de fenantrolina, e (d) 10, 50, 100 e 200 mM de hidroxilamina. Foram observados efeitos similares entre DCMU e fenantrolina, o valor da fluorescência inicial foi alterado, contudo a fluorescência máxima não se alterou, havendo o desaparecimento da fase de IP. Hidroxilamina interagiu e inibiu o complexo de evolução de oxigênio e causou desequilíbrio entre a taxa de redução Q_A pelo fotossistema II e a taxa de oxidação Q_A pelo fotossistema I.

Palavras-chave: DCMU, hidroxilamina, fenantrolina, complexo de evolução de oxigênio, fluorescência da clorofila

Introduction

Photosynthesis is a physical-chemical process by which plants, algae and some bacteria convert light energy into chemical energy. Photochemical events begin with the capture of photons by the photosystem II (PSII) antenna complex, where the excitation of the chlorophyll in the reaction center (P_{680}) causes the reduction of pheophytin (Pheo) forming P_{680}^+ and Pheo$^-$. A residue tyrosine (TyrZ) reduces P_{680}^+, receiving electrons from the oxygen evolving complex (OEC). Pheo transfers electrons to the primary electron acceptor (Q_A) which, in turn, gives them to Q_B, which can accept two electrons, leading to the formation of plastoquinol (PQH_2) (MSILINI et al., 2011).

PSII is a chlorophyll-containing membrane-bound enzyme that uses the energy of light to take electrons from water and reduces plastoquinona into a water/plastoquinone photo-oxidoreductase (CARDONA et al., 2012). Structurally, PSII reveals the transmembrane reaction core consisting of the heterodimeric D1/D2 proteins, which are centrally involved in binding the photo-oxidizable P_{680} chlorophyll complex as well as providing most of the ligands to the Mn_4Ca cluster that is the site of water oxidation (WILLIAMSON et al., 2011). These proteins have two bound plastoquinones, Q_A and

Q_B, which act as sequential electron acceptors of the PSII.

Chlorophyll (Chl) *a* fluorescence techniques are useful to monitor *in vivo* the changes of the photosynthetic apparatus in plants. The experimental procedure detects and evaluates such changes through their impact on functional photosynthetic properties. These techniques have the following advantages: provide an early diagnosis of vitality changes; can be used to screen not only leaves but any green part of the plant; are rapid; can be applied *in vivo* and *in situ*; can be carried out anywhere - in the field, greenhouse or laboratory; use small samples; are not invasive; are inexpensive (STRASSER et al., 2000, 2004; TSIMILLI-MICHAEL; STRASSER, 2008; ZHANG et al., 2013).

Chl *a* fluorescence signals have been extensively used for the assessment of several environmental impacts on photosynthetic metabolism. They allow the identification, separation and quantification of mechanisms that quench variable Chl *a* fluorescence emitted by PSII, which indicate not only changes in photosynthetic performance, but also allow the localization of primary sites of damage (GUIDI et al., 1997). The Chl *a* fluorescence analysis, also, allows to obtain detailed information about PSII electron transport.

Inhibition of PSII was also reported for several substances. Consequently, changing the flow of electrons caused by these inhibitors can be easily measured by monitoring the induction of Chl *a* fluorescence. Inhibition of electron flow is one of the main tools to elucidate and explain the mechanism of photosynthetic electron transport. Under these conditions, photosynthetic electron transport can be down-regulated to decrease electron transfer to oxygen. This regulation of photosynthetic electron transport depends on the components of the photosynthetic electron transport chain between PSII and PSI.

The DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] inhibits electron transfer from Q_A to the secondary quinone acceptor of PSII (Q_B), which results in a rapid reduction of Q_A and an increase in fluorescence as photochemical quenching is prevented. A slower increase in fluorescence follows, which is associated with the decay of nonphotochemical quenching (CHEMERIS et al., 2004; BOISVERT et al., 2006; BAKER, 2008). The addition of the herbicide DCMU induces a fast rise of the fluorescence yield during the first 2 ms of illumination; it transforms the regular OJIP

sequence into an OJ curve (STRASSER et al., 1995). DCMU displaces Q_B from its binding site at the D1 protein (VELTHUYS, 1981), and prevents the re-oxidation of Q_A^- by forward electron transport. This leads to a considerable simplification of the OJIP transient: the J and I steps disappear and the F_M is reached after ~2 ms (= J step) (TÓTH et al., 2005).

The phenanthroline ($C_{12}H_8N_2$) inhibits photosynthetic electron transport (SATOH, 1974; WRAIGHT, 1981) by competitive replacement of the secondary quinone acceptor Q_B from its binding site on the D1-protein, thus preventing the oxidation of reduced Q_A (KONDRASHIN et al., 1989). Chemeris et al. (2004) showed the inhibition of photosynthetic electron transfer with phenanthroline in *Chlorella*, which, at high concentrations, decrease the F_M due to suppression of the slow phase of fluorescence rise.

The hydroxylamine (NH_2OH – dihydridohydroxidonitrogen) inhibits the electron donation to P_{680}, nevertheless the acceptor site of the D1-protein can be damaged (BLUBAUGH et al., 1991). A electron donation rate from the water splitting complex to P_{680} can be affect by an appropriate concentration of hydroxylamine (CANAANI et al., 1986; KOMENDA et al., 1992; MEI; YOCUM, 1992; TERJUNG el al., 1997; DEWEZ et al., 2007; TRACEWELL; BRUDVIG, 2008). However, this inhibitor can act as an electron donor to PSII restored to the regular shape of the fluorescence rise (STRASSER et al., 2000)

In the present work, we investigated the effect of various inhibitors on the photosynthetic electron transport chain using Chl *a* fluorescence in canola leaf discs.

Material and methods

Plant material and growth conditions

Canola seeds were sown in pots with sand, and supplied with Hoagland solution. The plants were grown in greenhouse, (temperature between 18-25°C, photosynthetically active radiation between 400 – 500 μmol photons m^{-2} s^{-1}). Young expanding leaves, collected from greenhouse plants, were used to get the discs (10 cm^2).

Treatments

The leaf discs were incubated in the dark for 2 hours in 150 mL of the solution: a) water – control; b) DCMU at 500 μM; c) phenanthroline at 10 mM; and d) hydroxylamine (10, 50, 100 and 200 mM).

Chlorophyll a fluorescence measurement - JIP-test, normalization and subtractions of the transient fluorescence curves

Chl *a* fluorescence transients of dark-adapted leaf discs of canola plants were measured using a Handy PEA (Plant Efficiency Analyzer, Hansatech, UK). Discs (10 cm^2), kept in dark for at least 0.5-h in specially provided clips that fit onto the discs. The transients were induced by one flash with 3,000 μmol photons m^{-2} s^{-1}. The polyphasic fluorescence rise, OJIP, was measured during the first second of illumination. The quantification of the OJIP fluorescence transient is based on the polyphasic fast fluorescence rise from the lowest intensity F_0 (minimum fluorescence) to the highest intensity F_M (maximum fluorescence) (STRASSER; TSIMILLI-MICHAEL, 2001). The fluorescence intensities determined at 50, 100 and 300 μs ($F_{50μs}$, $F_{100μs}$ and $F_{300μs}$, respectively), 2 and 30 ms (F_{2ms} - F_J and F_{30ms} - F_I) and at F_M (maximum fluorescence) were used to calculate the JIP-test parameters (STRASSER; STRASSER, 1995). The intensity measured at 50 μs was considered to be the initial fluorescence (F_0). The plotted fluorescence values were the averages of 15 measurements (discs) of each treatment.

For comparison of the events represented by the OK, OI and IP phases, the transient curves were normalized as the relative variable fluorescence as: $W_{OK} = (F_t - F_0)(F_K - F_0)^{-1}$, $W_{OJ} = (F_t - F_0)(F_J - F_0)^{-1}$, $W_{OI} = (F_t - F_0)(F_I - F_0)^{-1}$ and $W_{IP} = (F_t - F_I)(F_M - F_I)^{-1}$ (YUSUF et al., 2010). For analysis of the differences kinetic, the differences between the relative variable fluorescence curves of the stress treatments and control were calculated ($\Delta W = W_{treatment} - W_{control}$); this procedure reveals bands that are usually hidden between steps O and P on the relative variable fluorescence.

Results and discussion

The use of inhibitors of photosynthetic electron transport chain is very useful for detailing the mechanisms involved in photosynthetic electron flow. Chl *a* fluorescence, though corresponding to a very small fraction of the dissipated energy from the photosynthetic apparatus, is usually accepted to provide an access to the understanding of its structure and function (STRASSER et al., 2000).

The D1-protein plays an important role in the electron transfer out of PSII. This protein can be damaged and lost, or bind other components than Q_B, like 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The consequence is a blocked electron transfer out of PSII and the accumulation of reduced primary quinone Q_A^- (TERJUNG et al., 1997).

A typical time course of fluorescence rise is characterized by O-J-I-P pattern, which can be only differentiated when the logarithmic time-axis is used. The J, I, and P steps appear at about 2, 20 and ~ 300 ms, respectively (STRASSER et al., 1995), as the accumulation of Q_A^- occurs at the position of the J step (LAZÁR, 1999), this step is the photochemical phase (STRASSER et al., 1995). Fluorescence changes during the fast part (between steps O and P) of the transient can be primarily correlated with the events taking place in the course of successive reduction of the electron acceptors of the entire photosynthetic electron transport chain. Figure 1 presents the fluorescence intensity and relative fluorescence variable, as $V_t = (F_t - F_0)(F_M - F_0)^{-1}$. In the Figure 1A and 1B demonstrate that the F_J value increases with use of the DCMU and phenanthroline, the treatments changed significantly the F_0 value, but de F_M did not change, compared to the control, suggesting full inhibition of PSII. The initial relative slope of the fluorescence rise from F_0 to F_J showed intensification, suggesting that the blocked electron transport rate from Q_A to Q_B resulted in Q_A accumulation.

The DCMU causes a limitation to the acceptor site of the electron transfer in PSII, preventing reoxidation of Q_A^- by intersystem electron transport (HSU, 1992; TÓTH et al., 2005). In this way, leaf discs under the action of the DCMU showed Chl *a* fluorescence kinetic as a single phase of induction, where F_M is reached after approximately 2 ms (J-step).

In this study, F_0 increased in the presence of DCMU, which could be described as a transformation of a part of the active PSII-centers into a form of silent reaction centers (RC) (non Q_A reducing reaction centers). The F_M values were the same in the presence or absence of DCMU. In the case of control leaf discs, the PQ pool is in its reduced state at the F_M level, whereas in the case of DCMU treated leaf discs, the PQ pool was oxidized. In the presence of DCMU, the re-oxidation of Q_A^- by forward electron transport was inhibited and F_M is reached shortly after the OJ phase, which is the photochemical phase of the Chl *a* fluorescence transient (TÓTH et al., 2005). This fact can be confirmed by the increase in F_J, and disappearance of the IP phase (Figure 1A). Similar effects were observed by replacing DCMU with phenanthroline.

Hydroxylamine is used as an irreversible inhibitor of the OEC (BULYCHEV; VREDENBERG, 2001), it inhibits the donation of electrons to P_{680} and can damage the D1 protein, and release the manganese on t he OEC; but at low
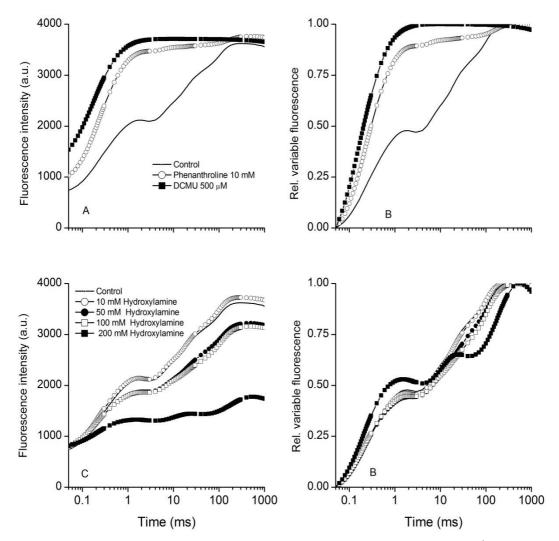
Figure 1. Chlorophyll *a* fluorescence transients (A, C) and relative variable fluorescence [$V_t = (F_t - F_0)(F_M - F_0)^{-1}$] (B, D) in canola leaf discs incubated in different solutions. (A, B) water (control), DCMU at 500 μM and phenanthroline at 10 mM; (C, D) water (control) and hydroxylamine at 10, 50, 100 and 200 mM.

concentrations, it can act as an electron donor to PSII (DEWEZ et al., 2007). The activation and inactivation of the OEC is a regulation mechanism under *in vivo* conditions determined by the way manganese is bound in the complex.

The use of hydroxylamine changed the typical shape of OJIP curves, principally at 200 mM (Figures 1C and D); and we observed that the F_J, F_I and F_M values decreased with increasing hydroxylamine-concentration, without changes in F_0 value. The inhibition of fluorescence slow rise indicates that the rate of Q_A reduction by PSII under these conditions is far below the rate of Q_A oxidation by PS I (BULYCHEV; VREDENBERG, 2001).

The Chl *a* fluorescence transients of the leaf discs with hydroxylamine were double normalized between F_0 (0.05 ms) and F_K (0.3 ms), expressed as $W_{OK} = (F_t - F_0)(F_K - F_0)^{-1}$ (graphic insert in Figure 2A). Subsequently, the control transient was

subtracted from the transients of the treated leaves ($\triangle W_{OK}$) (Figure 2A). The difference transients made the L-band visible (a peak around 0.15 ms). A positive deviation indicates the transformation of a sigmoidal fluorescence rise toward an exponential rise (STRASSER; STIRBET, 1998), with a decrease of energetic connectivity (or grouping) between PSII units. The response of the L-band was dose-dependent and indicated a decrease in energetic connectivity between PSII units at high doses.

The difference kinetics $W_{OJ} = (F_t - F_O)(F_J - F_O)^{-1}$ was also plotted in the 0.05-2 ms time range (Figure 2B graph insert), and then, the control transient was subtracted from the transients of the treated leaves ($\triangle W_{OJ}$) (Figure 2B). A change in the amplitude of the K-band (at 300 ms) was found with increase in the inhibitor, which indicates that hydroxylamine interacted and inhibited at OEC level.
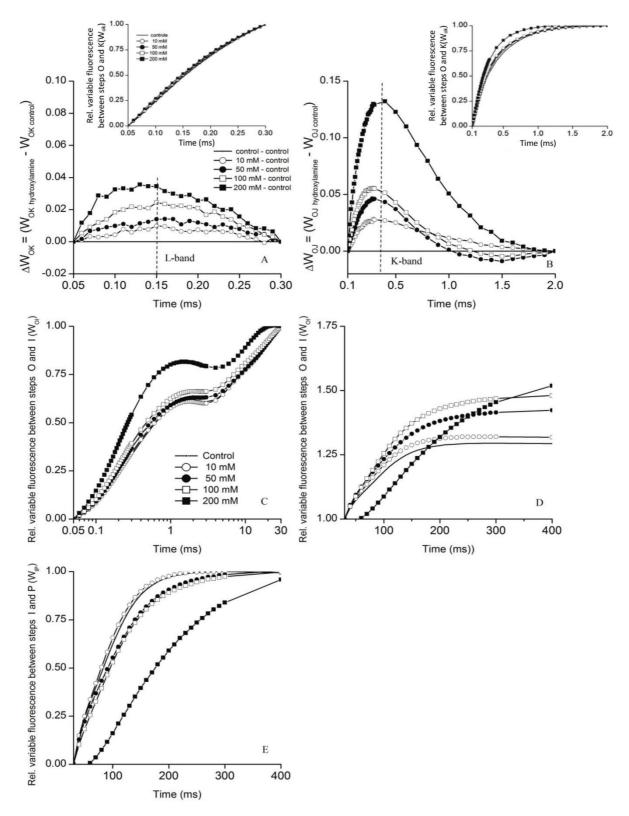
Figure 2. Chlorophyll fluorescence rise kinetics of canola leaf discs after incubation in hydroxylamine. (A) $\triangle W_{OK} = W_{OK\ hydroxylamine} - W_{OK\ control}$, insert graphic [$W_{OK} = (F_t - F_0)\ (F_K - F_0)^{-1}$]; (B) $\triangle W_{0J} = W_{OJ\ hydroxylamine} - W_{OJ\ control}$, insert graphic [$W_{OJ} = (F_t - F_0)\ (F_J - F_0)^{-1}$]; (C) Relative variable fluorescence between F_0 and F_I [$W_{OI} = (F_t - F_0)\ (F_I - F_0)^{-1}$] (D) Relative variable fluorescence between F_0 and F_I to $W_{OI} > 1$; (E) Relative variable fluorescence between F_I and F_M [$W_{IP} = (F_t - F_I)\ (F_M - F_I)^{-1}$].

The K step is usually hidden in the O–J rise, because under most conditions it does not present a sufficiently strong limitation on electron transport through PSII. A more pronounced K-band can be explained by an imbalance within PSII between the electrons, leaving the reaction centers at the acceptor side and the electrons donated by the donor side (STRASSER, 1997). The K-band can be observed in the fluorescence rise of, e.g. heat-stressed samples (TÓTH et al., 2005) and it becomes more pronounced in response to drought stress (OUKARROUM et al., 2007), and the appearance of the K-band coincides with a limitation on the donor side of PSII. It has been suggested that the appearance of the K step is caused by inhibition of OEC (STRASSER, 1997).

Figure 2C, where fluorescence data were normalized between the steps O (50 µs) and I (30 ms), presented as relative variable fluorescence $W_{OI} = (F_t − F_0)(F_I − F_0)^{-1}$ vs. time (logarithmic time scale from 0.05 up to 30 ms). This normalization serves to distinguish the sequence of events from exciton trapping by PSII up to plastoquinone (PQ) reduction (O–I phase; W_{OI} from 0 to 1) (YUSUF et al., 2010). It was observed that the leaves treated with 200 mM presented changes in W_{OI} compared with the others doses and control. This observation can be interpreted as the accumulation of Q_A^-, possibly due to a decrease in the electron transport beyond Q_A^- (HALDIMANN; STRASSER, 1999).

To analyze the I–P phase, two different normalization procedures were used:

a) the fluorescence data were normalized between the steps O and I ($W_{OI} = (F_t - F_0)(F_I - F_0)^{-1}$, as in Figure 2D, nevertheless herein only the part with $W_{OI} ≥ 1$ was plotted in the 30–400 ms time range (linear scale). This corresponds to the events from the PSI-driven electron transfer to the end electron acceptors on the PSI acceptor side ($NADP^+$), starting at PQH_2 (plastoquinol). For each curve, the maximal amplitude of the fluorescence rise reflects the size of the pool of the end electron acceptors at the PSI acceptor side (YUSUF et al., 2010). The increase in hydroxylamine concentration up 100 mM caused an increase in this pool size, except for the treatment with 200 mM, because the transient curve did not show a typical shape. This result allows us to evaluate the sequence of events for transferring electrons from plastoquinol (PQH_2) to the final electron acceptor of the PSI, confirming the imbalance between the rate of Q_A reduction by PSII and the rate of oxidation of Q_A by PSI;

b) fluorescence data were normalized between the steps I and P, as $W_{IP} = (F_t - F_I)(F_P - F_I)^{-1}$ (Figure 2E), this normalization enabled a comparison of the reduction rates of the end electron acceptors pool (YUSUF et al., 2010). The half-times are shown by the crossing of the curves with the horizontal dotted line drawn at $W_{IP} = 0.5$ (half rise). This permits the estimative of the overall rate constant (inverse of the half-time) of the processes leading to the reduction of the end electron acceptors (longer half-time corresponds to lower rate). Our results showed that the reduction rates of the end electron acceptors pool were lower (longer half-time) in the hydroxylamine concentrations of 50 and 100 mM compare with the control and 10 mM.

Figure 3A shows that the F_J, F_I and F_M values decreased with increasing hydroxylamine-concentration, but the treatments did not change the F_0 value, compared to the control; and at sub-saturating hydroxylamine concentrations, there was a variability in the extent of inhibition of the PSII. The relative fluorescence variable at J-step (V_J) was similar until 100 mM, but increased at 200 mM. However, the relative fluorescence variable at I-step (V_I) decreased in concentrations above 50 mM (Figure 3B).

From the analysis of the transients measured by JIP-test parameters (STRASSER; STRASSER, 1995; TSIMILLI-MICHAEL; STRASSER, 2008) we can deduce structural and functional parameters, quantifying the photosynthetic behavior of the samples. The presence of hydroxylamine allows us to demonstrate that various biophysical and JIP-test parameters were altered.

The energy fluxes per RC (Reaction Center), or also called specific energy fluxes are functional parameters, while the energy fluxes per ABS or flux ratios express, by definition, the corresponding quantum yields, which are structural parameters (STRASSER et al., 2000; 2004), These parameters have been derived on the basis of the energy flux theory (STRASSER, 1982).

In Figure 3C, we observed that the ABS RC^{-1} (measure for the average absorption per reaction center or the apparent antenna size, i.e. the amount of absorbing chlorophyll per fully active (Q_A^- reducing) reaction center) increased slightly when the leaves were treated with hydroxylamine until 100 mM, but the ABC RC^{-1} value was higher at 200 Mm. The other fluxes ($TR_0 RC^{-1}$, $ET_0 RC^{-1}$ and $RE_0 RC^{-1}$) showed no changes with hydroxylamine.

The maximum quantum yield of primary photochemistry ($\varphi_{Po} \equiv TR_0 ABS^{-1}$) and maximum yield of electron transport ($\varphi_{Eo} \equiv ET_0 ABS^{-1}$) decreased with increasing hydroxylamine

concentration (mainly at 200 mM); the quantum yield of electron transport from Q_A^- to the PSI end electron acceptors ($\varphi_{Ro} \equiv RE_0\,ABS^{-1}$) showed small increases up to 100 mM (Figure 3D).

The probability that a trapped exciton moves an electron into the electron transport hain further than Q_A^- ($\Psi_{Eo} \equiv ET_0\,TR_0^{-1}$) had a decrease at higher hydroxylamine concentration, and the efficiency
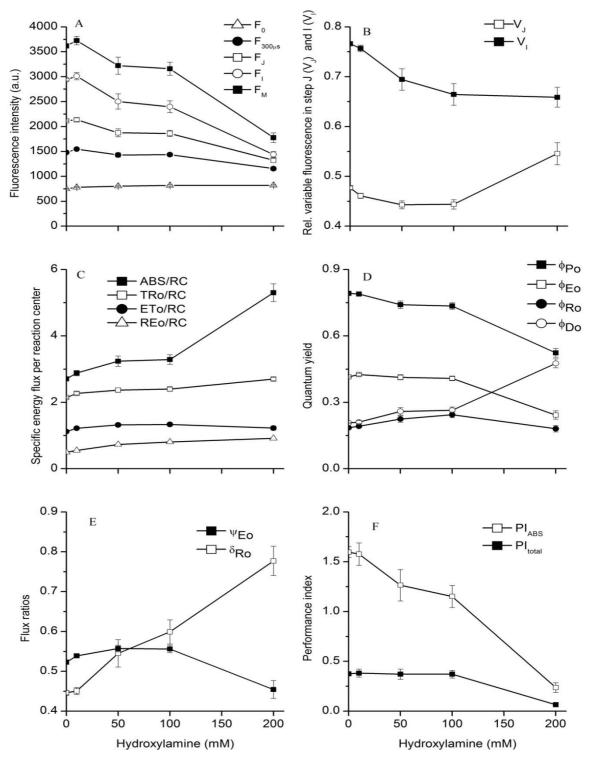

Figure 3. Fluorescence Intensity at steps O (50 µs) (F_0), K (300 µs) ($F_{300\,\mu s}$), J (2 ms) (F_J), I (30 ms) (F_I) and F_M (A); relative variable fluorescence between step O - J (V_J) and O – I (V_I) (B); specific energy flux per reaction center; (D) quantum yield; (E) flux ratios; (F) performance index calculated by the JIP-test.

with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors ($\delta_{Ro} = RE_0\ ET_0^{-1}$) increased with increasing hydroxylamine concentration.

The lowest values of maximum quantum yield of primary photochemistry ($TR_0\ ABS^{-1}$) at high hydroxylamine concentration indicate limitation in primary photochemical events (CHEN et al., 2011). And the decrease in the maximum yield of electron transport ($ET_0\ ABS^{-1}$) indicates decrease in electron transport beyond Q_A.

The Performance Index (PI_{ABS}), which is a combined measure of three partial performances: a) related with the amount of photosynthetic reaction centers, b) the maximal energy flux, which reaches the PSII reaction center, and c) the electron transport at the onset of illumination, was dose-dependent (decrease with increase of the dose) (Figure 3F). But, the Total Performance Index (PI_{total}), which measures the performance up to the PSI end electron acceptors, was constant up 100 mM, and then decreased.

Conclusion

The use of DCMU and phenanthroline provoke alterations in the value of initial fluorescence, but not of maximum fluorescence, and cause a limitation in the acceptor site of the electron transfer in photosystem II. The discs under of the DCMU show Chl *a* fluorescence kinetic as a single phase of induction, where maximum fluorescence is reached after approximately 2 ms, and the IP phase is not observed. Hydroxylamine, at high concentrations, inhibits the oxygen-evolving complex and causes an imbalance between the rate of Q_A reduction by photosystem II and the rate of Q_A oxidation by photosystem I.

Acknowledgements

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