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Influence of drought, high temperature, and carbamide cytokinin 4-PU-30 on photosynthetic activity of bean plants. 1. Changes in chlorophyll fluorescence quenching

I. YORDANOV, V. VELIKOVA, and T. TSONEV

Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 21, Sofia 1113, Bulgaria

Abstract

Fifteen-day-old bean plants (*Phaseolus vulgaris* L.) grown in a climatic chamber were exposed to water deficit (WD) and high temperature (HT) stresses applied separately or in combination. Changes in chlorophyll fluorescence quenching were investigated. Bean plants that endured mild (42 °C, 5 h for 2 d) WD separately or in combination with HT did not change their q_P and q_N quenching (measured at 25 °C) compared with those of the control. After 5 min testing at 45 °C, q_P in control and droughted plants strongly decreased, while q_P of plants that experienced combined WD+HT stress was insignificantly influenced, suggesting the acclimation effect of HT treatments. At more severe stresses (after 3 d-treatment), q_P measured at 25 °C was the lowest in WD+HT plants and q_N values were the highest. But when measured at 45 °C, q_P of WD+HT plants had practically the same values as at 25 °C. Under these conditions q_P of WD plants also showed an adaptation to HT. Twenty-four hours after recovery, the unfavourable effects of the stresses were strongly reduced when measured at 25 °C, but they were still present when measured at 45 °C. Positive effect of the carbamide cytokinin 4-PU-30 was well expressed only in droughted plants.

Additional key words: acclimation; *Phaseolus vulgaris*; treatment duration; water deficit.

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Fax: 359-2-73-99-52; e-mail: yordanov@obzor.bio21.bas.bg
Abbreviations: Chl – chlorophyll; F_0 – initial, F_v – variable, and F_m – maximal chlorophyll fluorescence; HT - high temperature; LHCP – light-harvesting chlorophyll *a/b* protein complex(es); PS2 - photosystem 2; 4-PU-30 – N-(2-chloro-4-pyridyl)-N-phenylurea; Q_A and Q_B – primary and secondary quinone acceptors of PS2; q_P and q_N - photochemical and non-photochemical Chl fluorescence quenching; RC – reaction centre(s); TM - thylakoid membrane; WD - water deficit; ΔpH – intrathylakoid proton gradient.
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Introduction

Water deficit (WD) and high temperature (HT) are among the most important environmental constraints that limit the rate of photosynthesis (Turner and Kramer 1980, Ingram and Bartels 1996). In the nature where various environmental factors normally change in unison and interact, combined stresses may elicit more pronounced effect than single stress in a controlled environment (Larcher *et al.* 1990). This conclusion is supported by Al-Khatib and Paulsen (1989) who show that thermal injury of *in vivo* photosynthesis is strongly aggravated by bright irradiance. Between environmental stresses an antagonistic interaction has often been found (Larcher *et al.* 1990, Havaux 1992).

Photosystem 2 (PS2), the most sensitive component of the photosynthetic system (Berry and Björkman 1980), plays a key role in the response of leaf photosynthesis to environmental perturbations (Chaves 1991). Dehydrated leaves retain a substantial part of their photosynthetic capacity (Cornic and Ghashghaie 1991). The combined action of WD and HT complicates the plant's response. Havaux (1992) demonstrated that the stability of PS2 to heat strongly increased in potato leaves exposed to WD. In addition, when plants were exposed to strong irradiance at high temperature, PS2 photochemistry was significantly less inhibited in dehydrated leaves than in the control, well hydrated leaves (Havaux 1992).

Analysis of chlorophyll (Chl) fluorescence at room temperature showed that PS2 of sorghum is fairly tolerant to imposed water stress. WD caused a slight decrease in efficiency of excitation capture (F_v/F_m) by open PS2 reaction centres (RC) (Cechin 1998). In droughted dark-adapted wheat plants no significant damage was found in the maximal efficiency of PS2 photochemistry, activity of PS2 RC, its oxidizing and acceptor sides, or in its antenna system. However, PS2 photochemistry in light-adapted leaves of WD plants was slightly modified as shown by a small decrease in the F_v/F_m. With increasing time and rate of desiccation (at 70 and 30 % relative air humidity, RH) the F_0 value in a moss increases, leading to a decrease in the F_v/F_p value (Bartošková *et al.* 1999). The damage to the acceptor side of PS2 dominating at 85 % RH is followed by a functional disconnection of P680 from the antenna system. A long-term drought stress on pea PS2 (reduction of water content by 35-80 %) led to a considerable depletion of PS2 core and the remaining PS2 complex was functional and reorganized, with a unit size (LHCP/PS2 core) two fold greater than that of well irrigated plants (Girardi *et al.* 1996). WD also caused an increase in phosphorylation of PS2 core and in D1 protein synthesis.

Stresses strongly influence the plant hormonal status (Morgan 1990). Application of cytokinins of purine (kinetin) and carbamide (4-PU-30) type favours protein synthesis and inhibits senescence of detached leaves. The 4-PU-30 [N-(2-chloro-4-pyridyl)-N-phenylurea] is a synthetic compound with biological properties qualitatively similar to those of cytokinins of the adenine type (Mok *et al.* 1987, Sudo 1994). We found (Yordanov *et al.* 1998) that plants which endured WD separately or in combination with HT recovered its O_2-evolving capacity better if they were sprayed with μM solutions of 4-PU-30. The aim of this investigation was

to establish the changes in photochemical and nonphotochemical fluorescence quenching (q_P and q_N) caused by WD and HT applied separately or in combination. We also attempted to find whether the stimulating recovery effect of 4-PU-30 on photosynthetic apparatus could be established by means of q_P and q_N.

Materials and methods

Plants: Young bean plants (*Phaseolus vulgaris* L.) cv. Cheren starozagorski were cultivated on sand with Knop's nutrient solution in a climatic chamber (temperature 23–25 °C, irradiance 120 μmol m^{-2} s^{-1}, 12 h photoperiod, relative air humidity about 70 %, and sand moisture 80 % of total capacity). After emergence of the first compound leaf the following variants were formed: control plants (C); control plants sprayed with 1 μM 4-PU-30 after the stressor had been removed (C+4-PU-30); plants that experienced WD by withholding irrigation (WD); plants that experienced WD and HT (WD+HT); plants that experienced WD and then were sprayed with 1 μM 4-PU-30 (WD+4-PU-30); plants that experienced combined stress and then were sprayed with 1 μM 4-PU-30 (WD+HT+4-PU-30).

The first two days the HT plants were treated for 5 h at 42 °C and on the third day for 2 h at 45 °C. On the 2nd day the water deficit in the leaves of droughted plants was between 6 and 9 % and in WD+HT plants between 14 and 16 %. On the 3rd day the water deficit was 18-22 or 40-42 %, respectively. Immediately after the stresses all plants were rewatered, sprayed with 1 μM 4-PU-30, and kept to recover for 24 h at 25 °C. After a 24 h recovery the water deficit in the leaves of droughted plants was 6-9 % and in WD+HT plants 18-22 %. The measurements were done on the primary leaves of bean plants.

Photochemical and non-photochemical Chl fluorescence quenching measurements: Kinetic analysis of Chl *a* fluorescence was performed to evaluate the q_P and q_N (Schreiber *et al.* 1986) using a pulse modulation Chl fluorometer *PAM 101-103* (*H. Walz*, Effeltrich, Germany). After 3 min dark adaptation, F_0, the initial Chl fluorescence yield under weak modulated irradiance (0.075 μmol m^{-2} s^{-1} photon flux density, PFD), and F_m, maximum total Chl fluorescence yield emitted during a saturating "white light" pulse (over 3000 μmol m^{-2} s^{-1} PFD, by *Schott KL 1500* radiation source), were determined. The leaf disc was then irradiated with continuous red radiation (125 μmol m^{-2} s^{-1} PFD). When the measuring radiation was applied, a modulation frequency of 1.6 kHz was used; otherwise the modulation frequency was set to 100 kHz. The same short pulses (with 20 s interval) of "white light" on the background of a red radiation were superimposed on F_v transient to determine the levels of F_v induced by saturating flashes [$(F_v)s$]. Photochemical (q_P) and non-photochemical (q_N) quenching parameters were calculated according to Schreiber *et al.* (1986). All parameters were measured at the temperatures 25 and 45 °C.

The experimental values were analysed statistically. The least significant difference (LSD) was used to evaluate differences between the variants according to Steel and Torrie (1960).

Results

The q_P quenching is a measure of the part of excitations captured by the open PS2 RCs and converted to chemical energy. The q_P reflects a capacity of RC to compete for Chl excited states and is related to the redox state of Q_A. But Chl fluorescence yield can also be lowered by mechanisms not directly related to the redox state of Q_A, *i.e.*, q_N. It includes mainly the "energy dependent" quenching (q_E) caused by intrathylakoid proton gradient (ΔpH), quenching related to "state1-state2" transition, and "photoinhibitory" quenching (Krause and Weis 1991). The dependence between ΔpH concentration in the thylakoid lumen and q_N is almost linear.

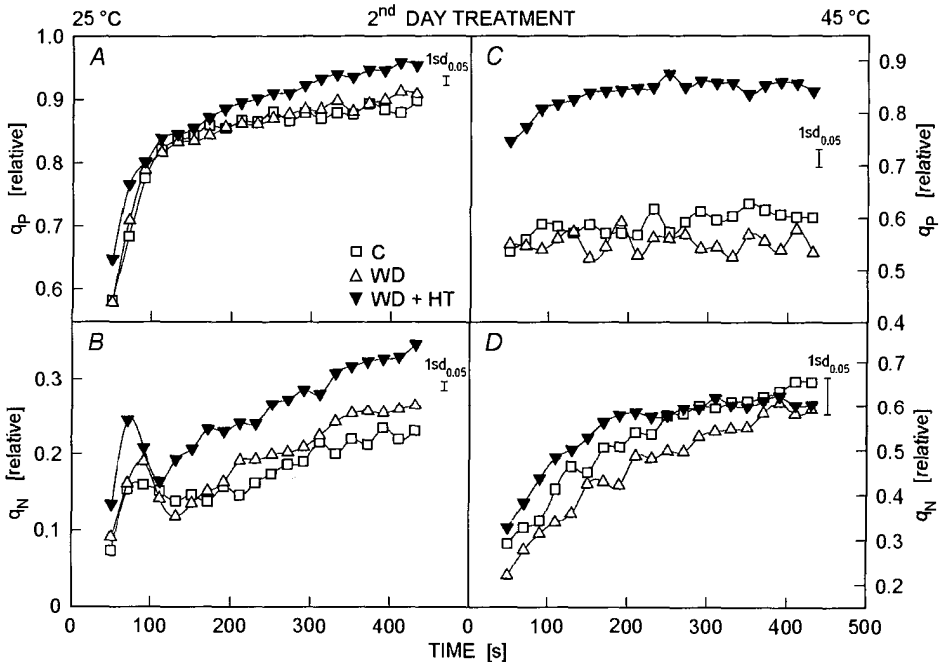

Fig. 1. Effects of mild water deficit (WD) applied separately or combined with mild high temperature (HT) of 42 °C, 5 h for 2 d on photochemical (q_P) and non-photochemical (q_N) quenching of chlorophyll *a* fluorescence of bean plants. The WD in plants that experienced only drought was 6-9 %, and in plants that endured a combined WD and HT stress 14-16 %. The measurements were done at 25 °C (*A, B*) or 45 °C (*C, D*).

The kinetics of q_P and q_N measured at 25 °C in control plants and plants droughted two days separately or in combination with mild HT (42 °C, 5 h each day) was determined (Fig. 1*A,B*). The first saturated radiation pulses caused significant enhancement of both q_P and q_N, after that either a plateau (or monotonous increase, WD+HT) was reached (q_P) or after weak decrease (q_N) up to 100-120 s a gradual increase followed, especially in plants under combined stress. The q_P increase reflects electron transport chain reoxidation and q_N an increase of proton gradient, *i.e.*, membrane energization. As photosynthesis starts in the induction period, the rate

of ATP consumption increases and ΔpH slightly decreases, causing a small drop in q_N. The increase in Q_A oxidation results from the acceleration of electron transport that occurs when the capacity of Calvin cycle increases. The rate of q_P appearance depends on the rate of synthesis of Calvin cycle intermediates (Krause *et al.* 1982). A low initial q_P in plants after WD or WD+HT may be due to a decrease in electron transfer from Q_A to Q_B (Schreiber *et al.* 1986, Govindjee 1990). The values of q_P (after the 200th s) and especially of q_N (120th s) in combined stress plants were higher than in control and WD plants. In all variants the q_P kinetics registered at 25 °C had two phases, fast and slow (Fig. 1*A*). The fast phase was probably connected with the Calvin cycle activation and the slow one was caused by additional pathway(s) that oxidize electron acceptors. The highest values of q_N at the latter part of its profile (Fig. 1*B*) indicate that leaves treated by mild heat attained a higher ΔpH dependent energy state of thylakoids than the corresponding control leaves.

When q_P and q_N of the above mentioned variants were measured at 45 °C, their kinetics were different (Fig. 1*C,D*) from those measured at 25 °C. First of all, the q_P in control and WD plants was much lower, its values were between 0.5-0.6 in almost all points of the measurement (Fig. 1*C*). In the WD+HT plants, however, the q_P values were about 0.8. The higher q_P in this variant was obviously connected with the acclimation effect of HT treatment. The almost complete absence of the first phase of q_P increase in control and droughted plants registered at 45 °C was probably connected with the destruction and increased permeability of thylakoid membranes (TMs) for protons.

The q_N measured at 45 °C showed a sharp increase up to the 240th s followed by a monotonous increase (control and WD) or plateau (WD+HT) reaching values of about 0.6 (Fig. 1*D*). In control plants these values were about three-fold higher than at 25 °C (see Fig. 1*B*). The increase in q_N under HT has been considered a protective effect, regulating radiant energy distribution in PS2 (Krause *et al.* 1988).

With decreasing WD the induction pattern, especially that registered at 45 °C, was markedly affected. At severe stresses (plants that experienced for 3 d drought separately or in combination with HT), the values of q_P at the first pulses were about 0.5 for WD and WD+HT plants and 0.6 for control plants (Fig. 2*A*). At about the 150th s (control and WD) or 200th s (WD+HT) a plateau was reached, being the highest in control plants and the lowest after the combined stress. In addition, while the plateau for the control and droughted plants occurred after the 4th saturated pulse, in WD+HT plants the plateau was registered after the 8th pulse. Though the kinetics of q_P in all three variants were similar, the q_P slope of WD+HT was lower than in the other two groups of plants.

Under the same conditions the q_N kinetics and its values in control and WD plants were rather different than in WD+HT plants (Fig. 2*B*). In the first two groups of plants the first saturated pulse caused a very low q_N which after weak increase at the 3rd-4th pulse showed a slight decrease followed by monotonous increase to the 400th s (0.25-0.30). Contrary, in the WD+HT plants the first three saturated pulses caused very strong (more than two-fold) increase of q_N and after the maximum at the 5th pulse a slight decrease and plateau were established. The highest q_N and the lowest

q_P in WD+HT plants indicated that the plants were fairly damaged. Obviously, such behaviour of photosynthetic apparatus may minimize the injury effect.

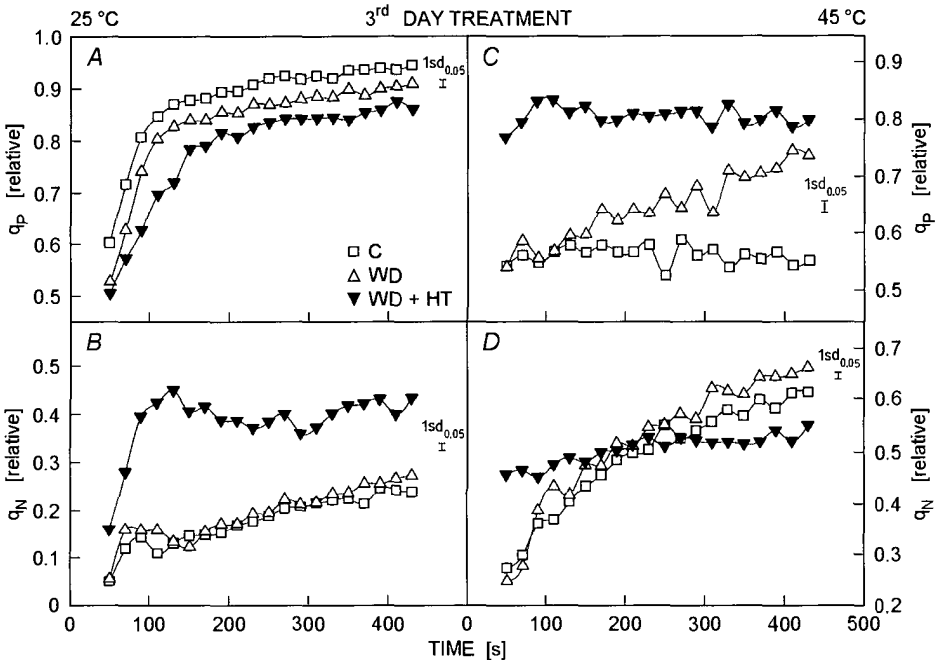

Fig. 2. Effects of severe water deficit (WD) applied separately or combined with high temperature (HT) of 42 °C, 5 h for 2 d + 45 °C, 2 h for 1 d on photochemical (q_P) and non-photochemical (q_N) quenching of chlorophyll *a* fluorescence of bean plants. The WD in plants that experienced only drought was 18-22 %, and in plants that endured a combined WD and HT stress 40-42 %. The measurements were done at 25 °C (*A, B*) or 45 °C (*C, D*).

The measurements of q_P of the same plant groups at 45 °C showed different kinetics and values similar to those registered at 25 °C (Fig. 2*C*). The lowest q_P values (between 0.5-0.6) in all points of measurement were observed in control plants. Under the same conditions the q_P quenching in WD plants monotonously increased from 0.55 (at the beginning) to 0.75 (at the end). The highest q_P values (about 0.8) were registered in WD+HT plants. After a slight increase to the 3rd saturated pulse a weak tendency to decrease was observed.

The q_N kinetics were different when measured at 45 °C on the 3rd day of treatment (Fig. 2*D*) compared with those on the 2nd day (Fig. 1*D*). While in the control and WD plants q_N strongly increased from about 0.25 to 0.60 (control) or 0.65 (WD), in the WD+HT plants the q_N kinetics did not change much (values between 0.45-0.55). Thus WD and especially WD+HT plants were more thermotolerant to very high temperature as a result of acclimation than the control plants.

Twenty-four hours after rewatering and temperature normalizing the unfavourable effects of the stresses were strongly reduced when measured at 25 °C (Fig. 3*A,B*) but they were still present when measured at 45 °C (Fig. 3*C,D*). In general, the character

of q_P kinetics at 25 °C of all variants was similar (Fig. 3*A*). Maximal values were reached at the 4-6th saturated pulses. Only a weak difference in the initial slope could be detected. It was steeper in control, WD+HT, and WD+HT+4-PU-30 plants.

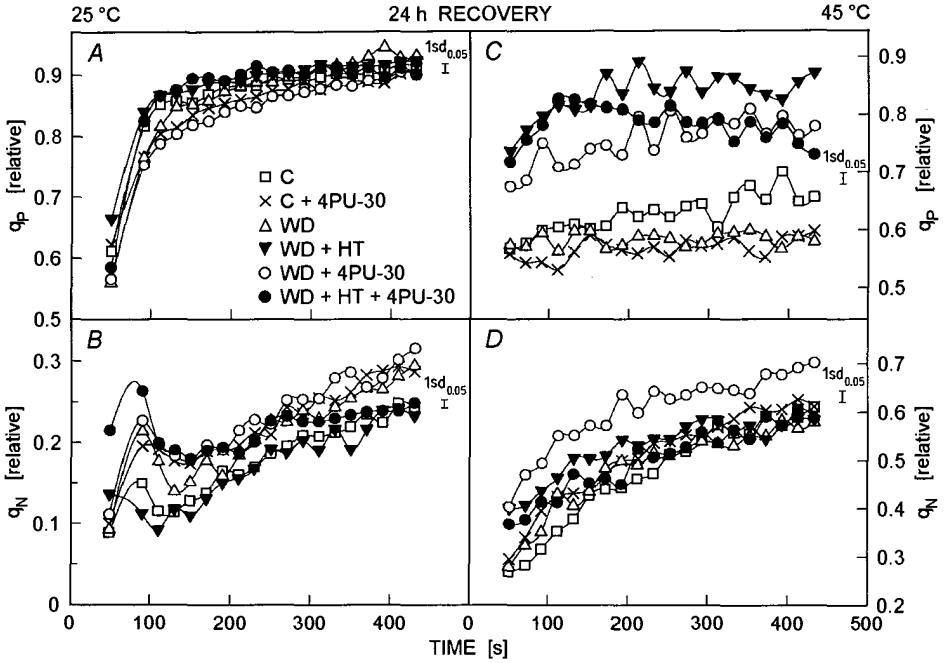

Fig. 3. Effects of severe water deficit (WD) applied separately or combined with high temperature (HT) of 45 °C, 5 h for 2 d + 45 °C, 2 h for 1 d on photochemical (q_P) and non-photochemical (q_N) quenching of chlorophyll *a* fluorescence of bean plants after a 24 h recovery. The WD in plants that experienced only drought was 6-9 %, and in plants that endured a combined WD and HT stress 18-22 %. The measurements were done at 25 °C (*A, B*) or 45 °C (*C, D*).

Under the same conditions the differences in q_N kinetics, especially in their initial slope were more evident. The q_N of WD+HT+4-PU-30 plants was the highest and that of WD+HT the lowest. In addition, at the beginning, the q_N values in plants after combined stress showed a weak decline followed by a monotonous increase.

Remarkable differences between the control and stressed plants were observed when the q_P and q_N were measured at 45 °C (Fig. 3*C*). The q_P values and the similar slopes for plants in variants WD+HT, WD+4-PU-30, and WD+HT+4-PU-30 were significantly different from those established for the other variants (Fig. 3*C*). The q_N kinetics measured under the same conditions were also different from those registered at 25 °C (Figs. 3*B,D*). In contrast with the q_N kinetics measured at 25 °C (Fig. 3*B*) those measured at 45 °C increased monotonously from the beginning till the end of their registration (Fig. 3*D*). The analysis of q_P quenching measured at 45 °C suggests that WD+HT, WD+4-PU-30, and WD+HT+4-PU-30 plants acquired an increased thermotolerance. After the 6 d recovery the results were similar to those obtained 24 h after rewatering the plants and their transfer to 25 °C. Their

photosynthesis was enough active and the differences between all variants were insignificant (values not shown).

Discussion

As Schreiber and Bilger (1987) emphasized for the Chl-protein complexes embedded in TMs, Chl *a* fluorescence is an intrinsic indicator for changes in membrane fluidity, stability, and organization induced by stress. Furthermore, the use of quenching analysis informs on stress induced changes in functions of TM-constituents and on the overall photosynthetic performance. The kinetics of q_P and q_N allow a better explanation of the dependence between the fluorescence yield and photosynthetic function, which is sensitive to WD and HT. The fluorescence measured at saturating pulses (F_v)s is particularly sensitive to many environmental stresses (Joshi *et al.* 1995). PS2 plays an important role in response and adaptation of photosynthesis to environmental stresses (Baker and Horton 1987). It is surprising, indeed, that PS2 shows a rather high resistance to WD which is reflected not only in the insignificant changes of steady-state fluorescence parameters but also in the characteristics of rapid fluorescence induction kinetics in WD plants (Cornic and Ghashghaie 1991, Cechin 1998). We found that bean plants that endured mild WD separately or in combination with HT did not change practically their q_P and q_N quenching characteristics measured at 25 °C compared with those of the control (Fig. 1). The high q_P associated with low q_N in control and stressed plants indicates efficient energy utilization by the Calvin cycle activity. These results agree with the findings of the above mentioned authors on the high stability of PS2 to water stress. But after a 5-min testing at 45 °C, q_P in control and droughted plants strongly decreased, while q_P of plants that experienced combined stress was only insignificantly influenced which suggested the acclimation effect of HT treatment. Nishiyama *et al.* (1993) proposed that the mechanism of photosynthetic acclimation to HT is related to the ability of O_2 evolving complex to protect against heat induced inactivation.

We also found that at more severe stresses the q_P values measured at 25 °C were the lowest and the q_N values were the highest in WD+HT plants (Fig. 2). But when measured at 45 °C, the q_P of WD+HT plants had practically the same values (reached after 120th s) as at 25 °C, about 0.8. Under these conditions WD plants also showed an adaptation to HT treatment, evaluated by q_P (Fig. 2*D*). These results agree with those of Havaux (1992) and Havaux and Tardy (1996) on detached leaves. Hence, the bean plants exposed to 45 °C maintained a higher proportion of oxidized to reduced Q_A, *i.e.*, had higher q_P values (the q_P coefficient reflects a capacity of RC to compete for Chl excited states and is related to redox state of Q_A) (Figs. 2*A* and 4*A*). According to Janssen *et al.* (1992), Q_A reduction is controlled by two factors: the rate of photochemical reduction of intersystem electron carrier pool, and the quantum distribution of excitation radiation within the photosynthetic apparatus that influences the balance of PS1 and PS2 activities.

In whole plants treated with HT and WD, PS2 is probably substantially more heat- and drought-tolerant than previously inferred from experiments with detached leaves and leaf discs. According to Joshi *et al.* (1995) low values of q_P in severely heat stressed leaves indicate that even a weak actinic irradiance can maintain about 80 % of photochemically active centres closed. This occurs since a severe heat stress (HS) may generate a large number of Q_B centres that are characterized with a slower rate of electron transfer from Q_A^- to Q_B (Q_B non-reducing centres) (Yordanov *et al.* 1997). During the WD and HT treatment of bean plants the CO_2 uptake is more inhibited than the primary PS2 photochemical activity (Yordanov *et al.* 1998). Since the dark and light reactions of photosynthesis are tightly coupled (Lawlor 1987), the lower CO_2 fixation activity decreases the demand for ATP and NADPH in the chloroplasts, which down-regulates photosynthetic electron transport system activity. The increased proton motive force cannot be dissipated in the stressed leaves as rapidly as in control leaves because the capacity for CO_2 assimilation is strongly reduced by the severe stress.

The heat effect is localized at both acceptor (Cao and Covindjee 1990) and donor sides (Katoh and San Pietro 1967). According to Joshi *et al.* (1995), though severe HT affected *in vivo* the acceptor side of PS2, the donor side remained relatively unaltered. Girardi *et al.* (1996) suggest that the syndrome caused by long-term WD on photosynthesis is a combination of at least two events: a reduction in the number of active PS2 RCs caused by a physical destabilization of the PS core, and PS reorganization with changed D1 turnover to counteract the core depletion.

Plants have evolved a number of adaptive mechanisms that allow the photochemical apparatus of photosynthesis to cope with rapid changes in irradiance, temperature, and water supply. Increased tolerance of PS2 to HT includes desiccation related accumulation of protective compounds; hence the rapid adjustment of PS2 heat resistance may involve a modification of the integration between violaxanthin and LHC of PS2 (Santarius and Müller 1979, Kaiser 1984, Havaux 1992, *etc.*). As a consequence the termoresistance of PS2 may be enhanced either directly (through conformational changes of PS2) or indirectly *via* carotenoid-dependent modulation of membrane lipid fluidity (Havaux and Tardy 1996). Xanthophylls epoxidized to zeaxanthin *in vivo* stabilize TM and protect thylakoids against heat induced disorganisation. This process preserves also their permeability.

Havaux (1992, 1996) proposed several possible mechanisms through which WD can protect against injury, *e.g.*, by strengthening the interaction between PS2 proteins and their lipid environment *via* alteration of the lipid composition of the TM or by accumulation of soluble protective compounds in chloroplast stroma under WD. In addition, WD counteracts the negative effects of high irradiance when combined with elevated temperature. Our results showed also an expressed positive effect of 4-PU-30 on droughted plants when testing was made at 45 °C.

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