UNIVERSITAT DE BARCELONA

FOTOSÍNTESIS, FOTOPROTECCIÓN, PRODUCTIVIDAD Y ESTRÉS ABIÓTICO: ALGUNOS CASOS DE ESTUDIO

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4. Photoprotection in water-stressed plants of durum wheat (*Triticum turgidum* var. *durum*): changes in chlorophyll fluorescence, spectral signature and photosynthetic pigments

4. Photoprotection in water-stressed plants of durum wheat (*Triticum turgidum* var. durum): changes in chlorophyll fluorescence, spectral signature and photosynthetic pigments

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Referencia bibliográfica del presente manuscrito

Tambussi EA, Casadesus J, Munné-Bosch S, Araus JL (2002) Photoprotection in water-stressed plants of durum wheat (*Triticum turgidum var. durum*): changes in chlorophyll fluorescence, spectral signature and photosynthetic pigments. *Functional Plant Biology* **29**: 35-44.

4.0. Abstract

We analysed the photoprotective response in water-stressed plants of durum wheat (*Triticum turgidum* L. *var. durum* cv. Mexa). The plants were grown in a greenhouse for 4 weeks and then exposed to water stress by withholding water for 8 days. Development of water stress was monitored as the decrease in relative water content (*RWC*) and net CO_2 assimilation of the last fully developed leaf. The photoprotective response was evaluated in the same leaves by analysing modulated chlorophyll fluorescence, leaf spectroradiometrical changes and pigment content. Measurements were performed 3, 6 (moderate stress) and 8 (severe stress) days after water-stress treatment began. The non-photochemical quenching of chlorophyll fluorescence (*qN*), as well as the contents of zeaxanthin and antheraxanthin increased significantly after 6 days of treatment. However, a further rise in these xanthophylls on day 8 was not associated with any increase in *qN*. In addition, the β-carotene content rose significantly on day 8, suggesting an increase in antioxidant defences. The photochemical index (*PI*), derived from spectroradiometrical measurements, showed a strong progressive drop on days 6 and 8, which was paralleled by an increase in the de-epoxidation state of the xanthophyll cycle (*DPS*), in particular by the zeaxanthin content. At midday, *PI* was strongly (negatively) correlated with *DPS* and *qN*. These results suggest that the photochemical index may be a reliable indicator of photoprotection in the study of plant stress, and in breeding programs.

Abbreviations used- A, antheraxanthin; Chl, chlorophyll (*a* + *b*); *DPS*, de-epoxidation state of xanthophyll cycle; *DW*, dry weight;, quantum yield of photosystem II; F_m and F_m', maximum fluorescence in dark-adapted and light-adapted leaves respectively; F_v, variable fluorescence in dark-adapted leaves; ΔpH, transthylakoidal gradient of pH; *PI*, photochemical index; *PPFD* photosynthetically-active photon flux density; *PSI* and *PSII*, photosystems I and II respectively; ϕ_{PSII}; *qN*, *qP* non-photochemical and photochemical quenching of chlorophyll fluorescence, respectively; *RWC*, relative leaf water content; V, violaxanthin; Z, zeaxanthin.

4.1. Introduction

Limitation of photosynthesis by water stress, especially when it is combined with conditions of high temperatures and light, may cause photo-oxidative damage to the photosynthetic apparatus if the plant does not avoid or dissipate the excess excitation energy (Smirnoff 1993; Asada 1999). In this case, water stress can damage several components of the cells, including proteins (He et al. 1995; Tambussi et al. 2000) and lipids (Moran et al. 1994). Among the mechanisms of energy dissipation, zeaxanthin dissipates the excess energy in chloroplasts via non-radiative processes (Demmig-Adams and Adams 1992). Non-radiative dissipation of energy in thylakoids, termed 'non-photochemical quenching' (*qN*), can be studied with modulated chlorophyll fluorescence of photosystem II (Krause and Weis 1991; Owens 1996). This mechanism is associated with the xanthophyll cycle, which consists of the de-epoxidation of the di-epoxide violaxanthin to zeaxanthin, and depends on the transtylakoid gradient of pH (ΔpH) in the chloroplast (formed in high light or stress conditions). In darkness, or when stress is removed, the reaction is reversed by zeaxanthin epoxidation (Yamamoto et al. 1962; Gilmore 1997). There is strong evidence that zeaxanthin (and perhaps monoepoxide antheraxanthin) formation is responsible for *qN* (see references in Gilmore 1997), which explains the photoprotective role of the xanthophyll cycle (Demmig-Adams and Adams 1992).

However, if the photoprotective mechanisms are insufficient, the leaves are protected from stress-induced oxidative damage by several antioxidant systems (Foyer 1994). Carotenoids, like β-carotene, are key scavengers of reactive oxygen species such as singlet oxygen, and so protect thylakoidal membranes from oxidative damage (Young 1991).

Changes in zeaxanthin (and antheraxanthin) content can be assessed in intact leaves by transmittance measurements (Yamamoto 1979; Bilger et al. 1989; Bilger and Björkman 1990; Mohanty et al. 1995) in the green band of the spectrum (505-510 nm) and, more recently, through reflectance measurements (Gamon et al. 1997; Filella et al. 1996). The mechanistic relationship between zeaxanthin (and antheraxanthin) content, *qN*, and absorbance changes near 500 nm was first shown by Gilmore and Yamamoto (1991). In this context, the spectral changes in the green region [as listed in '*Photochemical Reflectance Index*' after Gamon et al. (1990, 1992)], might be used to estimate zeaxanthin content and leaf photosynthetic performance. However, its reliability remains to be established. Whereas some studies have

shown a strong correlation between this index and the de-epoxidation state of the xanthophyll cycle [the relative proportion of the de-epoxidated *versus* the total content of the xanthophylls cycle involved in the cycle (Gamon et al. 1997)], others report much weaker correlation (Filella et al. 1996). In addition, little information is available on the use of the "photochemical index" in water-stressed plants, and particularly on its association with the photoprotective response of leaves.

Durum wheat is a widely cultivated crop around the Mediterranean basin, where it copes with fast-developing drought conditions (water stress combined with high radiation and high temperatures) during the last part of its cycle. In such a context, a finer understanding of the physiological mechanisms that allow the plant to resist photo-oxidative damage during drought, as well as the study of the reliability of fast-to-measure, non-destructive approaches to determine leaf photoprotection, may improve plant breeding. The aim of this study was to analyse the photoprotective response of water-stressed durum wheat plants, by near-simultaneous measurements of modulated chlorophyll fluorescence, spectral signature of leaves and pigment analysis.

4.2. Material and methods

4.2.1. Experimental set-up

Durum wheat seedlings (*Triticum turgidum* L. var. *durum* cv. Mexa) were grown at field capacity conditions in 6-L plastic pots (3 plants per pot) filled with peat and perlite [2:1 (v/v)], and fertilized with nutritive Hoagland´s solution (Hoagland 1937). Seedlings were cultivated under full sunlight during summer, in a greenhouse with cooling system at the Experimental Fields of the University of Barcelona (Barcelona, Spain). Average day/night temperatures and maximum photosynthetic flux density (*PPFD*) were 30/20ºC and 1400 µmol m^{-2} s^{-1}, respectively. Water-stress treatment was imposed on 4-week old plants by preventing watering. Control (irrigated) plants were maintained at field capacity throughout the experiment. The water content of the substrate was determined gravimetrically, and dropped to about 62%, 34% and 21% of that at field capacity after 3, 6 and 8 days of withholding water, respectively.

Water status, leaf gas exchange, modulated chlorophyll fluorescence, leaf spectral properties, and pigment content were determined at midday (between 12 am and 3 pm) in the youngest fully expanded leaf of plants. The diurnal course of the quantum yield of photosystem II (ϕ_{PSII}) photochemistry and photochemical index (*PI*) were also evaluated at 3-h intervals from morning to evening.

4.2.2. Leaf gas exchange and modulated chlorophyll fluorescence

Net CO_2 assimilation and stomatal conductance were measured with a portable gas-exchange system (LI- COR 6200, LI-COR Inc., Lincoln, NB, USA) at *in situ PPFD* conditions. Chlorophyll fluorescence parameters were determined with a modulated fluorimeter (Mini PAM Photosynthesis Yield Analyzer, Walz, Effeltrich, Germany). The maximum (F_m) and initial fluorescence (F_o) emissions were assessed in leaves after 30 minutes of dark adaptation, and the ratio of variable to maximum fluorescence (F_v/F_m) was then calculated as [(F_m - F_o)/ F_m]. F_s and F_m' (steady-state and maximum fluorescence yield in light-adapted leaves, respectively) were measured at *in situ PPFD* conditions. ϕ_{PSII} photochemistry was estimated as (F_m'- F_s) / F_m'. The parameter F_o' (minimum fluorescence yield in the light-adapted state) was estimated following Oxborough and Baker (1997). Photochemical (*qP*) and non-photochemical quenching (*qN*) of chlorophyll fluorescence were

calculated as described by Bolhár- Nordenkampf and Öquist (1993). Before fluorescence measurements, the instrument was set to zero to avoid interfering signals.

PPFD was measured with the sensor of fluorimeter clip, previously calibrated against a LI-COR sensor (LI-Cor Inc, Lincoln, NB, USA). The optical fibre in the leaf clip of the fluorimeter did not shadow the *PPFD* sensor.

4.2.3. Photochemical Index

Intact attached leaves were clipped by a small opaque chamber, consisting of two black-walled boxes articulated by a fringe, facing each other with a hole 6 mm diameter on the leaf side and a fibre optic connector on the opposite side. The upper part of the chamber was connected to an actinic light source with its own fibre optic (KL 1500 Electronic, SCHOTT GLAS, Mainz, Germany), where the internal filter restricting the spectra to the visible region was removed, in order to extend the range to the near-infrared region. The fibre optic tip was fixed 25 mm above the leaf and the *PPFD* on the leaf surface was around 400 μmoles m^{-2} s^{-1}. The lower part of the chamber was connected to a narrow-bandwidth visible/near infrared portable field spectroradiometer (FieldSpec UV/VNIR, from Analytical Spectral Devices, Inc., Boulder, Colorado, USA), through a fibre optic with a 25º field of view fixed 8 mm below the leaf surface. The spectroradiometer detects bands from 350 to 1050 nm wavelengths, thereby covering the visible and near-infrared portion of the spectrum. Transmittance spectra were obtained by dividing each wavelength of the spectra transmitted through the leaf by the reference spectra obtained in the same chamber without leaf. Photochemical index (*PI*) was then calculated as $(T_{531} - T_{570})/(T_{531} + T_{570})$, where T_n is transmitted light at a given wavelength [in nm (Gamon et al. 1997)].

4.2.4. Pigment determination

The apical half of leaves was collected after fluorescence and spectral measurements, immediately frozen in liquid nitrogen and stored at –80ºC until analysis. The extraction and analysis of pigments was carried out following Thayer and Björkman (1990) as described elsewhere (Munné-Bosch and Alegre 2000). In short, leaves were ground in a mortar in liquid nitrogen and extracted with cold 85% (w/w) acetone. After centrifugation for 3 min at 3ºC and 1000 *g*, the pellet was re-extracted twice with 100% acetone. The supernatants were combined

and filtered through a 0.20 µm syringe filter prior to analysis. Pigments were separated on a Dupont non-endcapped Zorbax ODS-5 µm column (250 x 4.6 mm, 20%C, Teknokroma, St. Cugat, Spain) at 30 ºC for 38 min at a flow rate of 1 mL min^{-1}. The solvents consisted of (a) acetonitrile/methanol (85: 15, v/v) and (b) methanol/ethyl acetate (68: 32, v/v). The gradient used was: 0-14 min 100% a, 14-16 min decreasing to 0%a, 16-28 min 0% a, 28-30 min increasing to 100% a and 30-38 min 100% a. Detection was carried out at 445 nm (Spectralphotometer 430 Kontron, Zurich, Switzerland). Calibration curves for chlorophyll *a*, chlorophyll *b*, lutein, β-carotene and zeaxanthin (*Z*) were established with authentic standards. Neoxanthin, violaxanthin (*V*) and antheraxanthin (*A*) were identified by their characteristic spectra, and their concentration was calculated from the corresponding peak area units to pmol per injection (Munné-Bosch and Alegre 2000). The de-epoxidation state of the xanthophyll cycle (*DPS*) was calculated as

$(Z + A)\ (V + A + Z)^{-1}$ following Gilmore and Yamamoto (1993) and Gilmore et al. (1998).

4.2.5. Water status

The relative water content (*RWC*) was calculated in the basal half of the leaves after the other measurements. For *RWC* measurements, leaf segments were weighted (w_i), floated on distilled water at 4°C overnight, weighted again (w_f) and dried at 80°C for 48 h, after which dry mass was determined (w_d). Relative water content was then calculated as:

$RWC = (w_i - w_d)\ (w_f - w_d)^{-1}$ x 100.

4.3. Results

Water-stressed plants showed a significant ($P < 0.05$) decrease in the relative water content (*RWC*) during the experiment, reaching values of 85% and 55% after 6 and 8 d of withholding water, respectively (Fig 1*A*). On day 3, although there were no significant differences in *RWC* between control and stressed plants, the latter showed a significant decrease in stomatal conductance (Fig. 1*B*). Indeed, water deficit strongly decreased the net CO_2 assimilation rate, which was almost abolished on day 8 (Fig. 1*C*). Total chlorophyll content and the ratio of chlorophyll *a* to *b,* did not show any difference between control and water-stressed plants throughout the experiment (Table 1). The quantum yield of photosystem II (ϕ_{PSII}, measured by the $\Delta F/F_m'$ ratio in light-adapted leaves) was significantly ($P < 0.05$) decreased in water-stressed plants at day 8 (Fig. 2*A*). Irrigated plants also showed a slight drop in the $\Delta F/F_m'$ ratio (Fig 2*A*), probably caused by the increase in *PPFD* (see the inset in Fig. 2*A*). However, the maximum yield of photosystem II photochemistry (measured by the F_v/F_m ratio in dark-adapted leaves) did not change in water-stressed plants (Fig. 2*B*). Non-photochemical quenching (*qN*) showed a significant ($P < 0.05$) increase on days 6 and 8 in water-stressed plants, compared with irrigated plants (Fig. 2*C*). This increase was nearly 50% on day 6 and it remained steady until day 8. The photochemical quenching (*qP*) of stressed plants, which represents the proportion of *PSII* reaction centres in an open (or oxidised) state, decreased significantly on day 8 (Fig. 2*D*).

In water-stressed plants, the "photochemical index" [*PI*, $(T_{531} - T_{570})/(T_{531} + T_{570})$] decreased by nearly 75% on day 8 (Fig. 3*B*), whereas in irrigated plants it remained unchanged throughout the experiment, with a value close to 0.02. When the measurements on control and stressed plants (either on day 6 or 8) were combined, *PI* showed a strong significant correlation ($r^2 > 0.7$) with *qN* (Fig. 3*C*), with a lower slope on day 8.

Figure 4 shows the midday carotenoid content throughout the experiment. Antheraxanthin and zeaxanthin were markedly higher on a chlorophyll content basis in water-stressed plants (Fig 4*A,B*). The antheraxanthin content started to increase on day 2 (although this increase was not significant until day 6). In contrast, violaxanthin decreased in water-stressed plants (Fig. 4*C*). The same pattern was observed when carotenoids were expressed on a dry weight basis (data not shown). As a result, the de-epoxidation state of the xanthophyll cycle (*DPS*) on day 8 increased by 185 % in stressed plants (Fig. 4*D*). However, the content of

other xanthophylls such as lutein and neoxanthin did not change during water stress (Fig 4*E*), whereas that of β- carotene increased strongly on day 8 (Fig. 4*F*).

The correlation between *DPS* and both *qN* (Fig. 5*A*) and PI (Fig. 5*B*) was significant ($r^2 \geq 0.7$). The slope of the regression plot for the relationship between *DPS* and *qN* decreased from day 6 to 8, similarly to that between *PI* and *qN* (Fig. 3*B*). The correlation of zeaxanthin and violaxanthin contents with *qN* and *PI* was also high, as was that of antheraxanthin ($P < 0.05$), although the latter was generally lower (Table 2).

Diurnal courses of ϕ_{PSII} and *PI* were studied as described above (Fig. 6). At the beginning of the treatment (day 3), no differences between irrigated and stressed plants were observed (Fig. 6*A*). However, ϕ_{PSII} decreased at midday in severely stressed plants, although this decrease was completely reversible at low *PPFD* (Fig 6*B*). *PI* showed moderate changes only in its diurnal course, in both irrigated and stressed plants (Fig. 6*C*). However, the *PI* of water-stressed plants decreased as treatment progressed, and showed a clear diurnal decline to zero in severely stressed plants (Fig. 6*D*).

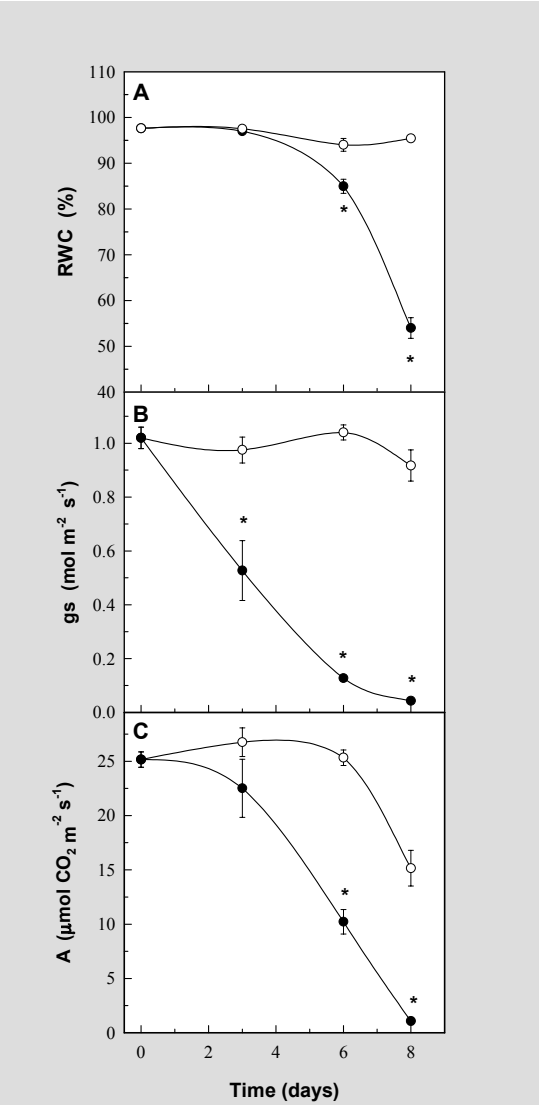

Figure 1. *Changes in relative water content (RWC, A), stomatal conductance (g_s, B) and CO_2 assimilation rate (A; C) in irrigated (open circles) and water-stressed (filled circles) plants. Day 0 was the last day before water withholding began. All measurements were performed at midday under natural sunlight. Each value represents the mean ± s.e. of five measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted with an asterisk.*

Table 1. *Total chlorophyll content [mg (chl a + b) g^{-1} DW], ratio of chlorophyll a to b and size of the xanthophyll cycle pool [mg (V + A + Z) g^{-1} DW] in control and water-stressed plants of durum wheat. Day 0 indicates initial conditions. Each value represents the mean ± s.e. of five measurements*.

Day		**Chl *a* + *b* content** *(mg g^{-1} DW)*	**Chl *a* / *b***	**Xanthophyll cycle** [*mg (V + A + Z) g^{-1} DW*]
0		14.09 ± 0.48	2.26 ± 0.05	0.64 ± 0.04
3	Control	11.94 ± 0.43	2.33 ± 0.05	0.57 ± 0.03
	Water stressed	12.24 ± 0.87	2.24 ± 0.09	0.61 ± 0.05
6	Control	11.78 ± 0.77	2.20 ± 0.05	0.60 ± 0.01
	Water stressed	12.81 ± 0.63	2.25 ± 0.06	0.62 ±0.03
8	Control	11.51 ± 0.62	2.17 ± 0.07	0.55 ± 0.04
	Water stressed	11.20 ± 0.58	2.24 ± 0.02	0.62 ± 0.03

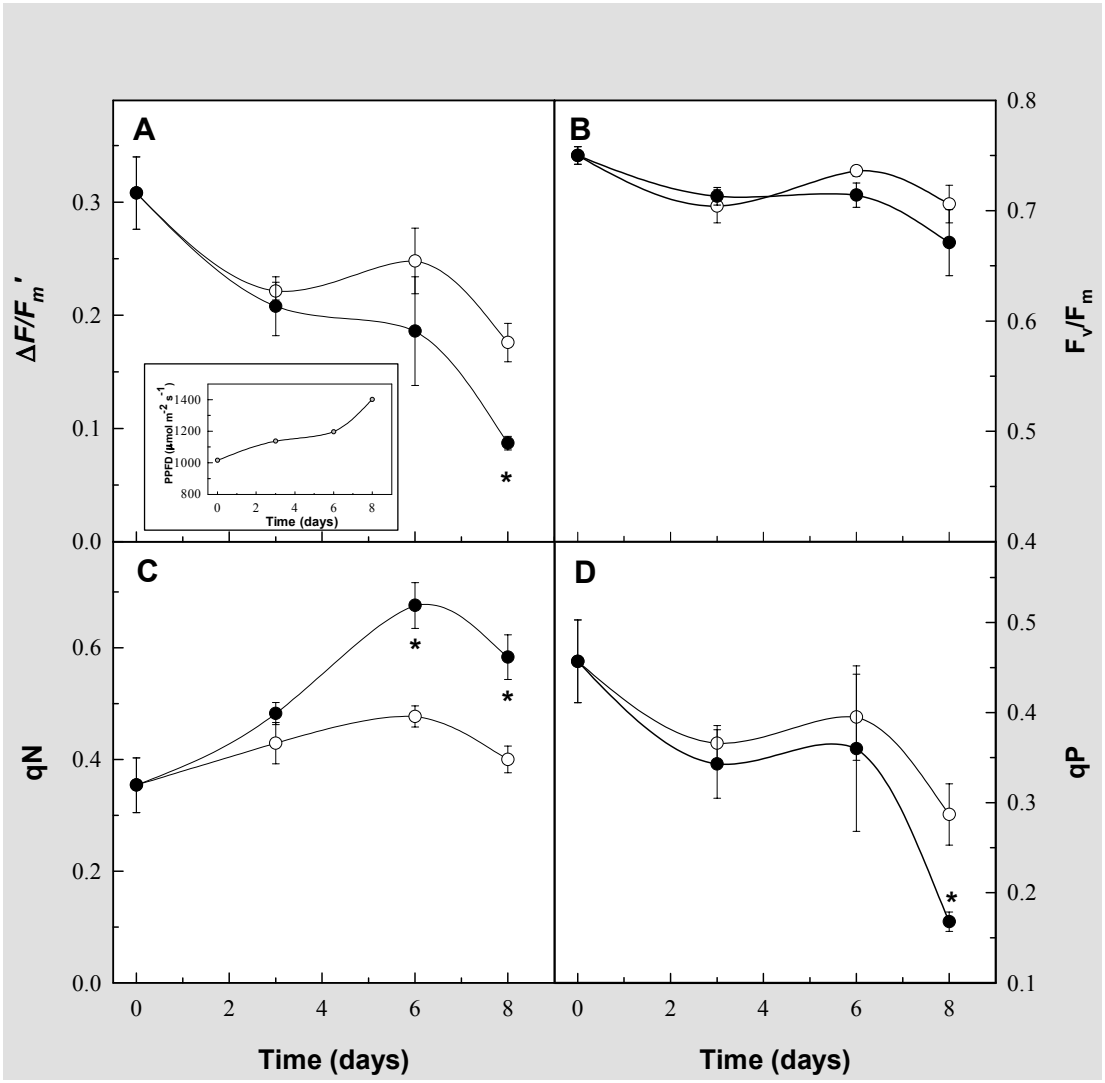

Figure 2. *Changes in actual quantum yield of photosystem II ($\Delta F/F_m'$, A), potential quantum yield of photosystem II (F_v/F_m, B), non-photochemical quenching (qN, C) and photochemical quenching (qP, D) in irrigated (open circles) and water-stressed (filled circles) plants. Day 0 was the last day before water withholding began. All measurements were performed at midday under natural sunlight. Each value represents the mean ± s.e. of five measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted with an asterisk.*

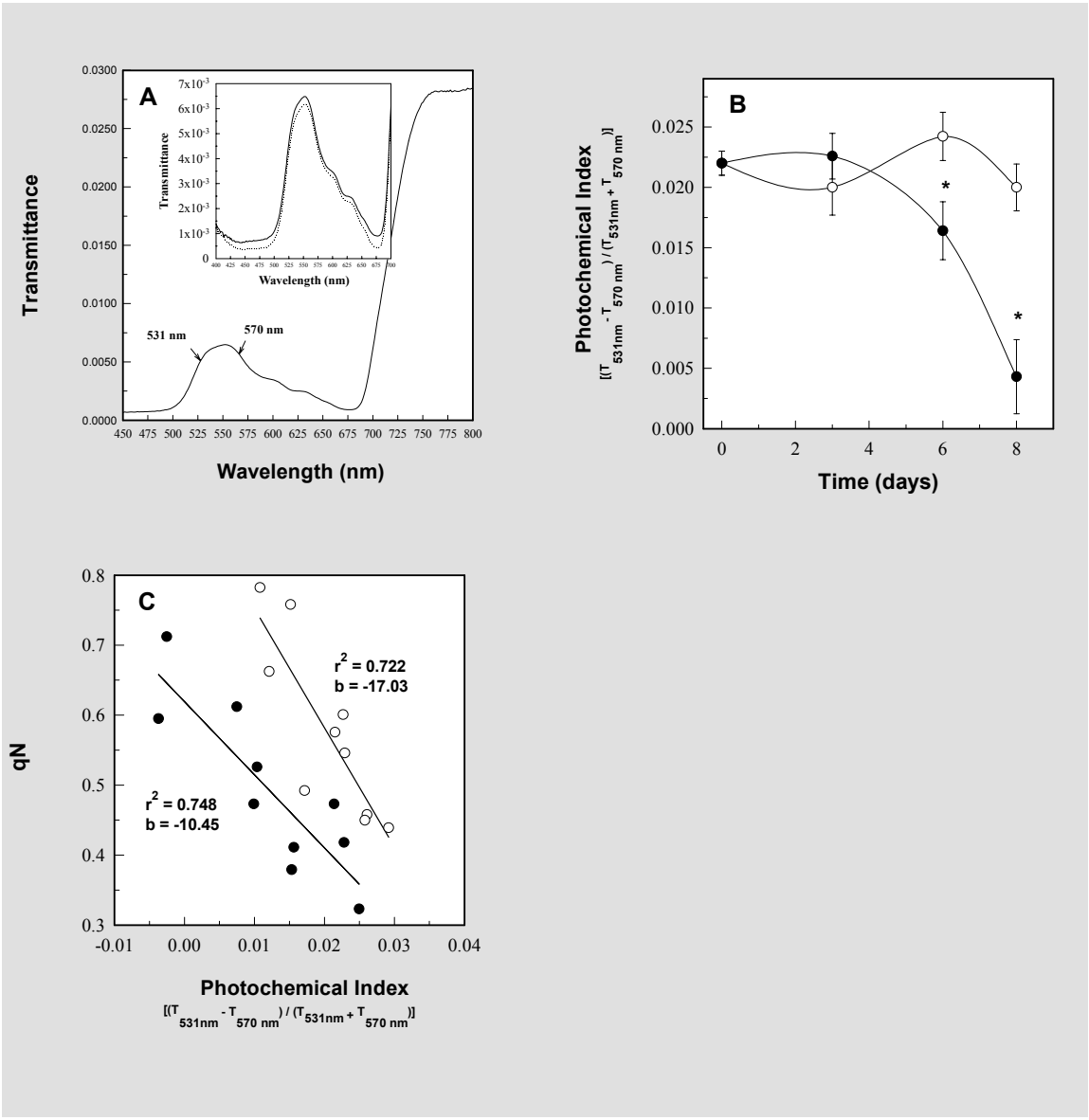

Figure 3. *Typical transmittance spectrum of durum wheat leaves (A). The arrows indicate the 531 and 570 nm wavelength bands used in calculating the photochemical index. Inset: transmittance spectrum in irrigated (RWC of 95 %, solid line) and water-stressed (RWC of 55 %, dotted line) conditions (B) Changes in the photochemical index (PI) of irrigated (open circles) and water-stressed (filled circles) plants. The index was calculated using the transmittance spectrum of leaves as* $(T_{531}-T_{570})$ */*$(T_{531} + T_{570})$*. Day 0 was the last day before water withholding began . All measurements were performed at midday under natural sunlight. Each value represents the mean ± s.e. of five measurements. Significant differences* $(P \leq 0.05)$ *between treatments according to the LSD test are denoted with an asterisk. (C) Correlation between PI* $[(T_{531}-T_{570})/(T_{531} + T_{570})]$ *and non-photochemical quenching (qN). Each point represents the mean of the 3 measurements performed on an individual leaf in the irrigated or water-stressed plants on days 6 (open circles) and 8 (filled circles). For each correlation, the determination coefficient* (r^2) *and the slope of the fitting line (b) are provided within the figure.*

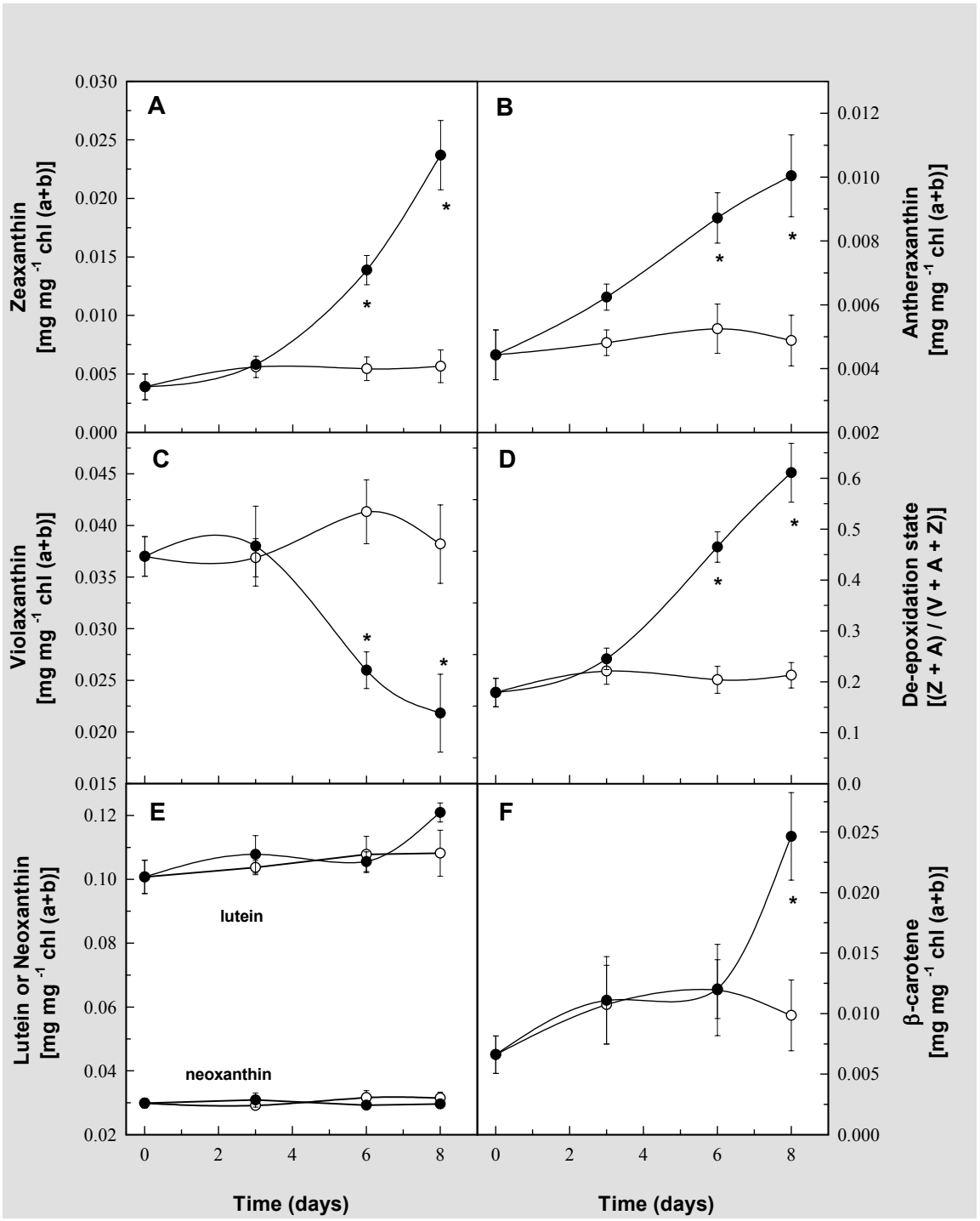

Figure 4. *Changes in the contents of zeaxanthin (A), antheraxanthin (B), violaxanthin (C), the de-epoxidation state of the xanthophyll cycle (D) and in the contents of lutein and neoxanthin (E) and β-carotene (F) in irrigated (open circles) and water-stressed (filled circles) plants of durum wheat. Carotenoid contents are expressed per unit weight of total chlorophyll [chl (a + b)], whereas the de-epoxidation state was calculated as (Z + A) / (V + A + Z). Day 0 is the last day before water was withheld. All measurements were performed at midday under natural sunlight. Each value represents the mean ± s.e. of five measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted with an asterisk.*

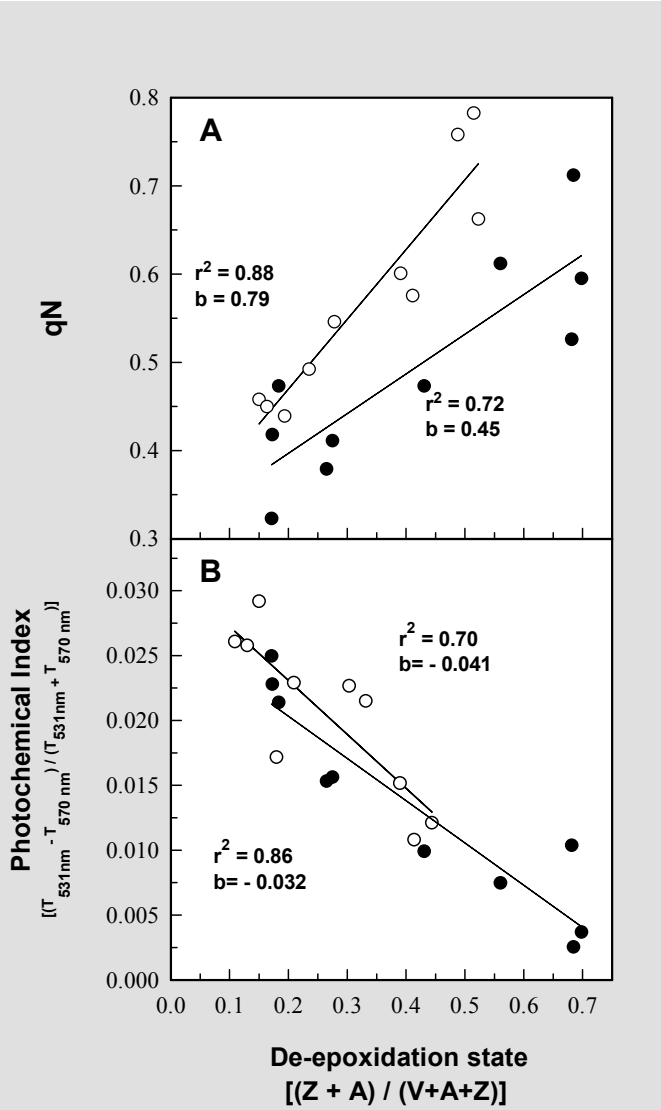

Figure 5. *Correlation between the de-epoxidation state of the xanthophyll cycle [(Z + A)/(V + A + Z)], and both non-photochemical quenching (qN; A) and the photochemical index [(T_{531}-T_{570})/(T_{531} + T_{570}); B]. Each point represents the mean of the three (transmittance) or one (carotenoid) measurements performed on an individual leaf in the irrigated or water-stressed plants on days 6 (open circles) and 8 (closed circles). Correlations were calculated on days 6 and 8. For each correlation, the determination coefficient (r^2) and the slope of the fitting line (b) are provided within the panels.*

Table 2. *Determination coefficients (r^2) of the correlation between non-photochemical quenching (qN) and photochemical index (PI) with zeaxanthin (Z), antheraxanthin (A) or violaxanthin (V) content on a dry weight basis. The r^2 of the correlations between the de-epoxidation state (DPS) of the xanthophyll cycle [(Z + A) / (V + A + Z)] and both qN and PI are also given for comparison purposes. In all cases, r^2 corresponds to the correlation between these parameters on days 6 and 8.*

	Day 6		**Day 8**	
	PI	**qN**	**PI**	**qN**
mg Z g^{-1}DW	0.63	0.83	0.9	0.75
mg A g^{-1}DW	0.39	0.77	0.45	0.27
mg V g^{-1}DW	0.77	0.79	0.67	0.66
DPS	0.70	0.88	0.86	0.72

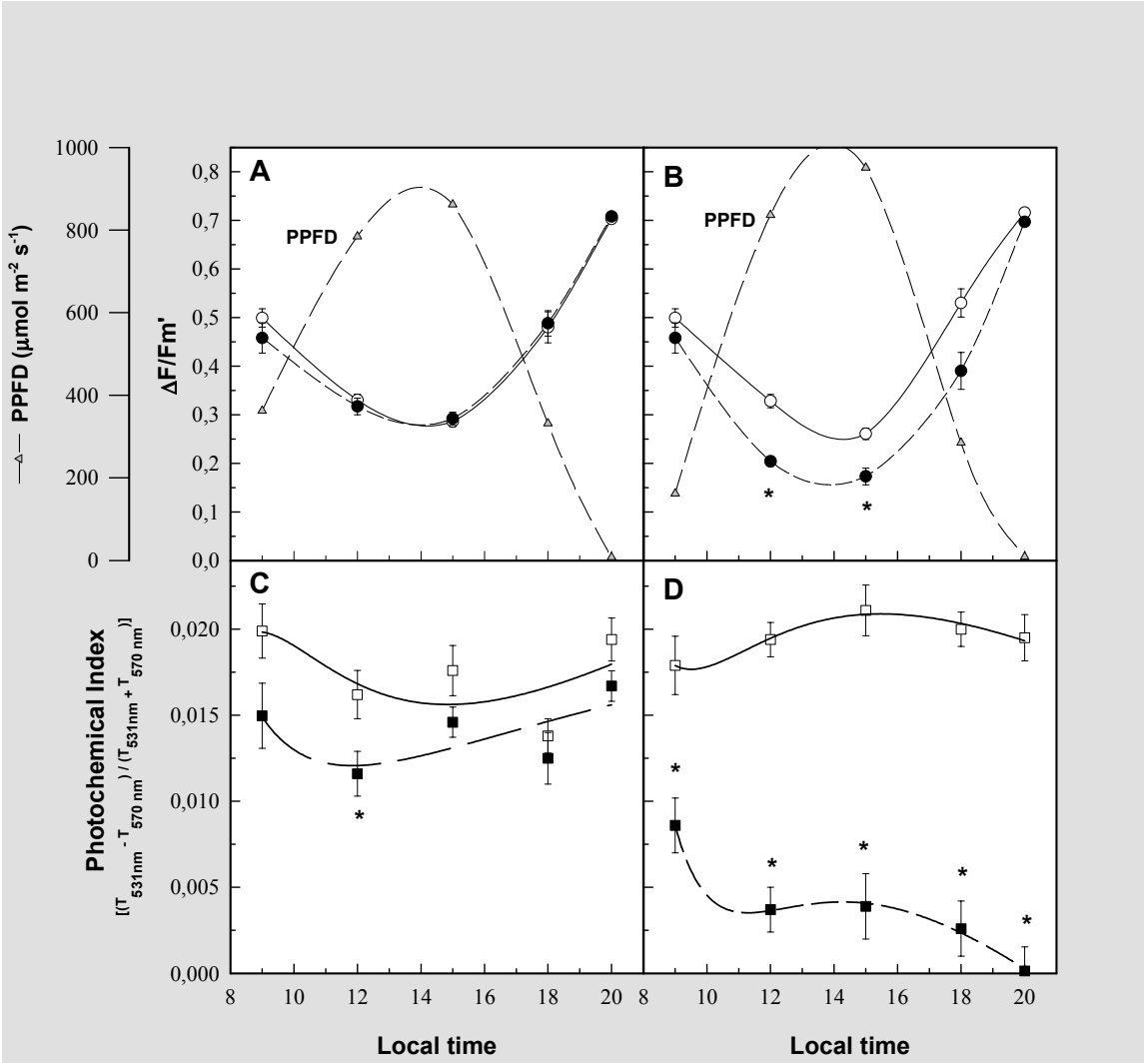

Figure 6. *Diurnal course of actual quantum yield of photosystem II (ΔF/Fm') (A, B) and the photochemical index [(T531-T570)/(T531 + T570); C, D) in irrigated (open symbol) and water-stressed (filled symbol) plants of durum wheat. The daily course of incident PPFD during the measurements is also shown in panels A and B. Panels A and C correspond to incipient stress (day 3, no significant differences in RWC between control and stressed plants). Panels B and D correspond to severe stress (day 8). RWC in irrigated and water-stressed plants was 97% and 62%, respectively. Each value represents the mean ± s.e. of five measurements. Significant differences (P ≤0.05) between treatments according to the LSD test are denoted with an asterisk.*

4.4. Discussion

4.4.1. Photoprotective response

Water-stressed plants showed a substantial decrease in the quantum yield of photosystem II (ϕ_{PSII}) paralleled by the drop in CO_2 assimilation rates (Figs. 1C, 2A). This reduction was associated with an increase in non-radiative dissipation (measured as *qN*) and a decrease in the proportion of open (oxidised) centres (measured as *qP*) (Figs 2C, D), in agreement with previous reports (Lu and Zhang 1998). Therefore, our results show substantial levels of saturation of the electron transport system, and thus excess of light in water-stressed plants, as reported elsewhere (see references *in* Cornic and Massacci 1996). Decrease in ϕ_{PSII} was reversible in the short-term, since the potential quantum yield (evaluated as F_v/F_m) was unaffected in water-stressed plants throughout the studied period (Fig. 2*B*). Nevertheless, as recent reports show that a considerable fluorescence signal in modulated fluorimeters may originate from *PSI* (Agati et al. 2000; Gilmore et al. 2000), interpretation of results is still open to discussion.

The increase in non-radiative dissipation was associated with a strong rise of zeaxanthin content, especially during moderate and severe stress. It is now well established that the changes in *qN* are linked to the dissipation as heat of excess absorbed light energy, in which the acidification of thylakoid lumen and the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin seem to play a key role (see references in Gilmore 1997). The increase in zeaxanthin content was very strong in our study (nearly by 380% during severe stress; Fig. 4*A*). Interestingly, the increase in zeaxanthin content between days 6 and 8 was not associated with an additional rise of *qN*. Indeed, the decrease between day 6 and 8 in the slope of the *DPS*-*qN* correlation (Fig. 5A) would support the opposite theory. Although this observation seems surprising at first, beyond a certain level, zeaxanthin accumulation does not promote a larger non-photochemical quenching capacity, because a limited number of xanthophylls molecules are involved in the *qN* mechanism (Hurry et al. 1997). Some reports show that the zeaxanthin content increases the non-photochemical quenching only at subsaturating ΔpH, and not at saturating ΔpH (see references in Neubauer 1993). The *npq2* mutant of *Arabidopsis* (with high *DPS* values) does not show *NPQ* values greater than the wild type (Niyogi et al. 1998). These observations are consistent with the model proposed by Gilmore et al. (1998), in which a specific binding site for a few zeaxanthin (or antheraxanthin)

molecules among, or in, the inner antenna of *PSII* is activated by a low intratylakoidal pH that increase the affinity for these xanthophylls. This was recently confirmed by Heber and coworkers (Bukhov et al. 2001), who showed that a few molecules of zeaxanthin per reaction centre is sufficient for effective thermal dissipation. In summary, our results are consistent with (and fully explained by) the saturable nature of the xanthophyll cycle pigments.

Despite the involvement of zeaxanthin in *qN* mechanisms, recent reports have shown that this xanthophyll also behaves as direct antioxidant (Götz et al. 1999). Here, severe water stress markedly increased the content of β-carotene, a key antioxidant in thylakoid membranes (Young and Britton 1990). Under severe water stress, the amount of reactive oxygen species increases in several species (Smirnoff 1993). Thus, in response to this oxidative stress, the β-carotene content increases in *Triticum aestivum* leaves (Bartoli et al. 1999). Therefore, our results indicate that β-carotene (and perhaps zeaxanthin) may directly protect thylakoid membranes under severe water stress, with a *RWC* of nearly 50% and CO_2 assimilation near zero on day 8. The rise in these carotenoids may reflect a requirement for increased protection of lipids and proteins, which may be oxidised in water-stressed plants (Moran et al. 1994; Tambussi et al. 2000). There is evidence that the xanthophyll cycle protects chloroplast membranes from photo-oxidation by a mechanism other than non-photochemical energy quenching, and that the presence of zeaxanthin itself in the thylakoid membranes would enhance the tolerance of thylakoids to lipid peroxidation (Havaux et al. 2000).

Water stress did not significantly alter the content of other xanthophylls, such as lutein and neoxanthin (Fig. 4*E*), which agrees with previous reports on other species (Logan et al. 1998; Verhoeven et al. 1999). In addition, there were no changes in the chlorophyll *a/b* ratio or in the size of the xanthophyll cycle pool (Table 1). This suggests that the increase in zeaxanthin and antheraxanthin is mainly explained by violaxanthin de-epoxidation, and not by *de novo* synthesis.

Finally, the changes in the content of carotenoids may be sequential. Incipient stress (day 3) moderately (although non-significant) increased antheraxanthin content (the first step in zeaxanthin formation), followed by a progressive rise in *qN*-related antheraxanthin and zeaxanthin contents. Finally, severe stress increased the β-carotene and zeaxanthin contents that were not associated with any further rise in *qN*.

4.4.2. Photochemical index, non-photochemical quenching and pigment relationship

PI measured at midday decreased sharply throughout the experiment (Fig. 3*B*). This drop was strongly correlated with the *qN* and *DPS* of the xanthophyll cycle (Figs. 3*C*, 5*B*). The correlation with antheraxanthin was less distinct (Table 2), suggesting that the change was mainly associated with zeaxanthin increase (Fig 4*A*). Under moderate stress, *PI* showed minor midday decreases in the diurnal course (Fig. 6*C*), although in severely water-stressed plants, the *PI* value dropped to zero at the end of the light period (Fig. 6*D*). The weak response in the diurnal course of *PI* in control and moderately stressed plants was unexpected. However, this observation could be explained because the more important changes in *DPS*, and subsequently in *PI*, take place at sunrise. Similar profiles, with an almost 100% change between 7 and 9 h, have been reported (Gamon et al. 1992). However, other studies describe a strong association of *PI* with both *PPFD* and ϕ_{PSII} (Gamon et al. 1997), in disagreement with our results. Gamon et al. (1997) reported that the PI is complex and has two components, one wavelength at 526 nm and another at 545 nm. The 545 nm wavelength saturates at low *PPFD* (near 100 μmol m^{-2} s^{-1}) and it may be linked to the chloroplast conformational changes associated with the build-up of the pH gradient. Nevertheless, Li et al. (2000) recently reported that the changes in the 535 nm light scattering (545 nm in other studies) absorbance are caused by the pH-dependent conformational changes in the PsbS protein.

Only the first wavelength (526 nm) is directly involved in the violaxanthin de-epoxidation and the zeaxanthin content, which are saturated at high *PPFD*. The distinct role proposed for the two spectral components of *PI* may partially explain the lack of uniform response of this index in several studies on various species and under various experimental conditions.

The small change in *PI* during the day suggests that *DPS* remains steady throughout the day. In non-senescent rice leaves, Murchie et al. (1999) observed that the *DPS* is constant and low in irrigated plants (15-20%, similar to midday *DPS* values of our control plants) during the light period. *PI* (measured at the canopy level) strongly varied in response to water stress, regardless of the epoxidation state profile (Gamon et al. 1992). Such a decrease in the *PI* of water-stressed plants was attributed to the large diurnal changes in canopy structure associated with severe midday wilting (Gamon et al. 1992). We did not encounter this methodological problem, because spectral transmittance was analysed on individual leaves.

Although the presence of artifacts in the spectral transmittance caused by the low *RWC* in the leaves cannot be ruled out, the normalised character of the index may minimise this possibility.

In our study, *PI* was formulated using the 531 nm band. Changes in this wavelength have been associated with several physiological parameters, such as radiation-use efficiency (Filella et al. 1996; Peñuelas et al. 1995), ϕ_{PSII} (Peñuelas et al. 1998; Gamon et al. 1997) and obviously the xanthophyll cycle (Filella et al. 1996; Peñuelas et al. 1995, Peñuelas et al. 1998; Gamon et al. 1997). However, none of these studies contrasted *PI* with *qN*, the parameter related to the xanthophyll cycle. We showed a strong correlation in midday measurements between *PI*, and both *DPS* and *qN*.

Further research is necessary to elucidate the pattern of response of *PI* under water stress and to evaluate its efficacy when assessing the photoprotective response of plants to other stresses. This index may be used to assess the photosynthetic use efficiency at the leaf or canopy level in a similar way to the fluorescence indicators of the photochemical efficiency of photosystem II and it may prove especially useful in breeding programs (Slafer et al. 1999).

Acknowledgements.- *We thank the "Servei de Camps Experimentals" and "Serveis Científico-Tècnics de la Universitat de Barcelona" for their valuable help, and Hoffman-La Roche (Dr. José María Hernández) for kindly providing us with carotenoid standards. This study was supported by the CICYT research projects PB97-0865 and AGF99-0611-C03-03 (Spain). E. Tambussi was the recipient of a fellowship from the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET, Argentina).*

4.5. References

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