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

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6. Is C_4 metabolism involved in the better photosynthetic performance of ears than flag leaves of durum wheat under drought?

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6.0. Abstract

The photosynthetic characteristics of the ear and flag leaf of well-watered (*WW*) and water-stressed (*WS*) durum wheat (*Triticum turgidum* L. var. *durum*) were studied in plants grown under greenhouse and Mediterranean field conditions. Gas exchange measurements simultaneously with modulated chlorophyll fluorescence were used to study the response of ear and flag leaf to CO_2 and O_2 during photosynthesis. C_4 metabolism was identified by assessing the sensitivity of photosynthetic rate and electron transport to oxygen. In addition, the histological distribution of Rubisco protein in ear parts was studied by immunocytochemical localisation. Relative water content (*RWC*) and osmotic adjustment (osmotic potential at full turgor) were also measured in these organs.

Oxygen sensitivity of the assimilation rate and electron transport, and the lack of Rubisco compartmentalisation in the mesophyll tissues indicated that C_4 metabolism does not occur in the ear of *WW* or *WS* plants. Nevertheless, photosynthetic activity of the flag leaf was more affected by *WS* conditions than that of the ear, under both growing conditions. The lower sensitivity under water stress of the ear than of the flag leaf was linked to higher *RWC* and osmotic adjustment in the ear bracts and awns. We also found a strong (negative) correlation between the water content (in fresh weight basis) of the several organs studied (leaf blades, glumes, lemmas and awns) in *WW* plants and the *RWC* of the same organs under *WS* conditions. This result suggests that xeromorphic traits (as well as osmotic adjustment) of bracts and awns is also related to ears' drought tolerance.

We concluded that the better performance of the ear under water stress (compared to the flag leaf) is not related to C_4 photosynthesis. Rather, drought tolerance of the ear is explained by its higher *RWC* in drought. Osmotic adjustment and xeromorphic traits of ear parts may be responsible.

Abbreviations used: A_{sat}, light-saturated net CO_2 assimilation rate; A^*_{sat}, corrected light-saturated net CO_2 assimilation rate of the ear (i.e. $A_{sat} + R_d$); F_m and F_m', maximum fluorescence in dark-adapted and light-adapted organs respectively; F_v/F_m, maximum efficiency of *PSII* photochemistry after dark-adaptation; F_v'/F_m', efficiency of energy capture by open *PSII* centres; F_o', minimum fluorescence yield in light-adapted state; ϕ_{PSII}, relative quantum yield of *PSII* photochemistry; *PPFD*, photosynthetically active photon flux density; q_P, photochemical quenching of chlorophyll fluorescence; R_d, dark respiration; *RWC*, relative water content; ψ_w, leaf water potential.

6.1. Introduction

Durum wheat is one of the more widely cultivated crops in the Mediterranean basin, where drought is the main abiotic stress limiting its production (Royo et al. 1998). Mediterranean climate is characterised by a progressive increase in drought (combination of water stress, high temperature and excess radiation) during late spring, coinciding with the grain filling of cereal crops (Acevedo et al. 1999). Filling of grains under these conditions is sustained (apart from the assimilates stored before anthesis in the stem) by the current photosynthesis of the upper parts of the plant, i.e. the flag and penultimate leaves plus the ear (Carr and Wardlaw 1965).

In the past, flag leaf photosynthesis was considered the main source of assimilates for grain filling (Evans et al. 1980), even though the function of ear photosynthesis in productivity of C_3 cereals had been under discussion for years (Kriedemann 1966). However, nowadays it is accepted that ear photosynthesis makes a major contribution to final grain yield (Simmons 1987; Araus et al. 1993a), and even more so under drought conditions when the ear may become the main photosynthetic contributor to filling of grains (Evans et al. 1972; Bort et al. 1994; Sánchez-Díaz et al. 2002).

Apart from the fact that the ear is closer than the leaves to the photosynthetic sink (i.e. the grains), the ear is a greater source of assimilates because its photosynthetic performance is better under stress. Several traits of the ear have been suggested as explanations for this. These can be summarised as (i) some degree of C_4 metabolism (either constitutive or drought-induced) and/or (ii) more xerophytic features (including anatomy and osmotic adjustment). The existence of some degree of C_4 metabolism in the ear parts of C_3 cereals has been reported (or at least suggested) in several studies (Nutbeam and Duffus 1976; Singal et al. 1986; Ziegler-Jöns 1989; Imaizumi et al. 1990). However, other studies have denied this possibility for durum wheat growing in absence of water stress (Araus et al. 1993b; Bort et al. 1995). The discrepancies between these studies could arise from the methodology used (for instance ^{14}C labelling in detached *versus* intact organs) and the environmental conditions of plant growth. Hence, C_4 metabolism in the ear might be induced only as response to stress (*e.g.* drought), but this hypothesis has not been examined.

Morpho-physiological traits of the ears, such as its xeromorphic anatomy (Grundbacher 1957; Araus et al. 1993a) and osmotic adjustment (Morgan 1980), could have important adaptive advantages under drought conditions. In fact, these characteristics may help the ear maintain under drought better water status and thus more photosynthetic activity than the flag leaf (Xu and Ishii 1990). However, to date just how these xerophytic adaptations explain the better photosynthetic performance of the ear under drought has not been extensively studied.

The aim of this study was to compare the photosynthetic characteristics and water status of durum wheat ear and flag leaf in response to drought during grain filling under greenhouse and Mediterranean conditions. In this context, whether C_4 photosynthesis existed in the ear in absence of stress or its induction by drought was assessed.

6.2. Material and Methods

6.2.1. Growing conditions

Two sets of experiments with plants grown either in greenhouse or under field conditions were run.

Greenhouse experiment. Durum wheat seeds (*Triticum turgidum* L. var. *durum*) cv. Korifla were grown at field capacity conditions in 3-L plastic pots (one plant per pot) filled with peat-perlite-vermiculite 2:1:1 (v/v) and fertilised with nutritive Hoagland solution. Plants were cultivated in a greenhouse in the Experimental Fields at the University of Barcelona (Barcelona, Spain). Mean day/night temperatures and maximum photosynthetic flux density (*PPFD*) were *ca.* 28ºC, 20ºC and 1500 μmol m^{-2} s^{-1}, respectively. All plants were maintained at container capacity until heading. At this juncture, water was withheld. In water-stressed (*WS*) plants, the water content of the substrate was determined gravimetrically and was maintained by sub-irrigation at *ca*. 30% of container capacity. Well-watered (*WW*) plants were maintained at container capacity throughout the experiment.

We measured gas exchange and modulated chlorophyll fluorescence of the ear and the flag leaf three times: at anthesis, and 15 and 30 days after anthesis. In addition, water status and osmotic adjustment were measured during grain filling.

Field experiment. This was also conducted at the Experimental Fields of the University of Barcelona. The experimental design was a randomised block (4.5 m^2 per plot) with three replications per treatment (well-watered and water-stressed). Conventional farming techniques were used. Durum wheat seeds of the same cultivar as above were sown in mid-February and the plots were periodically irrigated until anthesis. Then water stress (*WS*) was imposed by halting irrigation, whilst preventing rainfall with a transparent polyvinyl chloride shelter. The shelter was only used during rainfall episodes. The control (*WW*) plots continued to be irrigated throughout grain filling. Mean day/night temperatures and maximum photosynthetic flux density (*PPFD*) during grain filling were ca. 25ºC, 19ºC and 1950 μmol m^{-2} s^{-1}, respectively. Environmental conditions were monitored by a weather station (Delta-T Devices, Cambridge, UK) situated 8 m from the experimental plots.

Gas exchange, chlorophyll fluorescence and water status were measured on both organs 25 days after anthesis.

6.2.2. Water status determinations

6.2.2.1. Relative water content

Relative water content (*RWC*) was measured in ear parts (glumes, lemmas and awns) and in the blades of the flag leaf and the three leaves following the flag leaf from the top. Measurements on *WW* and *WS* plants in the greenhouse experiment were taken 20 days after anthesis. Leaf blade segments or ear parts were weighed (w_i), floated on distilled water at 4°C overnight, weighed again (w_f), and dried at 80°C for 48 h, after which dry mass was determined (w_d). Relative water content was calculated as: $RWC = (w_i - w_d)\ (w_f - w_d)^{-1}$ x 100.

6.2.2.2. Water potential

Water potential (ψ_w) was measured in the flag leaf of *WW* and *WS* plants from both the greenhouse and field experiment. Measurements were taken at midday, in anthesis and 15 (about mid-grain filling) and 30 days after anthesis, using a pressure chamber (ARIMAD-2, ARI Far Charuv-Water Supply Accessories, Israel) with a damp piece of paper at the bottom of the chamber to avoid excessive evaporation.

6.2.2.3. Osmotic adjustment

Osmotic potential of the flag leaf blade and ear parts (glumes, lemmas and awns) was determined at full turgor (Morgan 1984) in *WW* and *WS* plants of the greenhouse experiments 20 days after anthesis. Blades and ear parts were floated on distilled water at 4°C overnight, after which the samples were frozen in liquid nitrogen and stored at -30ºC until analysis. Before the measurements the samples were warmed at room temperature. Extraction was with a 1-ml syringe and the osmotic potential was measured with a VAPRO 5520 osmometer (Wescor INC., USA). The instrument was calibrated at standards of 100, 290 and 1,000 mmol Kg^{-1} (Wescor Inc. USA). Osmotic adjustment was defined as the difference of osmotic potential at full turgor between *WW* and *WS* plants (Babu et al. 1999).

6.2.3. Photosynthetic measurements

6.2.3.1. Gas exchange

Leaf gas exchange was measured, using an open IRGA LI-COR 6400 system (LI-COR Inc., Lincoln, NB, USA), at the flag leaf and the ear between 09:00 and 17:00, with a mixed sequence across treatments to reduce the bias due to timing. The temperature of the photosynthetic chambers was maintained at ca. 25ºC throughout the measurement period. The photosynthetic rate in the flag leaf blade was assayed with the standard cuvette (LI 6400-02). Gas exchange measurements in the ear were made with the Conifer Chamber (LI 6400-05), supplemented with a 500 W halogen lamp (model 64702, OSRAM S.A., Madrid, Spain). The lamp was placed about 30 cm above the ears and 7 cm of deionised water and 1 cm of Plexiglas in the container filtered the radiation. The lamp supplemented a *PPFD* of about 500 μmol m^{-2} s^{-1}. Plants were also irradiated with 500 μmol m^{-2} s^{-1} of *PPFD* for at least 30 min prior to photosynthesis measurements.

Dark respiration rate of the ear (*Rd*) was determined by darkening for at least 15 minutes. In the ear, corrected photosynthetic rate (A^*_{sat}) was calculated as (A_{sat} + *Rd*)**,** where A_{sat} is the light-saturated net CO_2 assimilation rate (Araus et al. 1993a).

6.2.3.2. Chlorophyll fluorescence

Steady-state modulated chlorophyll fluorescence of the flag leaves and the ear was measured by means of a modulated chlorophyll MiniPAM fluorimeter (Waltz, Effeltrich, Germany) used simultaneously with gas exchange measurements. The leaf cuvette of infrared gas analyser (LI-COR 6400) was modified to accept the optical fibre from the modulated MiniPAM fluorimeter, as previously described (Nogués and Alegre 2002). For the ear, the optical fibre was introduced in the conifer chamber and obliquely oriented over the ear. The fluorescence signals were analysed as described by Andrews et al. (1993) to provide estimates of the relative quantum yield of *PSII* photochemistry [ϕ_{PSII}, given by (F_m' - F_s) / F_m'], the efficiency of energy capture by open *PSII* centres [F_v'/F_m', given by (F_m' - F_o') / F_m'] and the photochemical quenching [*qP*, given by (F_m' - F_s) / (F_m - F_o')]. F_s and F_m' are the steady-state and maximum fluorescence yield, respectively, in light-adapted leaves and F_m is the maximum fluorescence in dark-adapted leaves. The parameter F_o' (minimum fluorescence yield in the

light-adapted state) was calculated following Oxborough and Baker (1997). The maximum quantum yield of *PSII* photochemistry [F_v/F_m, given by $(F_m - F_o)/F_m$, where F_o is the initial fluorescence in dark-adapted leaves] was determined in flag leaf and ear after 15 minutes of dark adaptation (Nogués et al. 1998).

6.2.3.3. Low oxygen conditions

To examine whether the decreases in CO_2 assimilation caused by water stress were related to differential effects on photorespiration and carbon assimilation metabolism, we inhibited photorespiration in the ear and the flag leaf by reducing the O_2 concentration of the atmosphere in which the photosynthetic parameters were measured from 21 to 2% (Nogués and Alegre 2002). Air at 2% O_2 and 98% N_2 was pumped to the air inlet port of the LI-6400. CO_2 was regulated with the CO_2 mixer of the LI-6400. Measurements at low oxygen conditions were made 15 and 30 days after anthesis.

In addition to these measurements of CO_2 assimilation rate in non-photorespiration conditions, we measured the response of ϕ_{PSII} to oxygen at several CO_2 concentrations. The change of ϕ_{PSII} was plotted *versus* CO_2 concentrations at both normal (atmospheric) and low (2%) oxygen.

6.2.4. Immunocytochemical localisation of Rubisco

6.2.4.1. Fixation and embedding

Flag leaves, glumes, lemmas and awns of *WW* and *WS* plants were sampled 20 days after anthesis. Cross-sections, 1-2 mm wide, were fixed at reduced pressure in 2% (v/v) paraformaldehyde, 0.2% (v/v) glutaraldehide in 0.1 M sodium phosphate buffer (pH 7.4) for 24 hours at 4ºC. The samples were subsequently rinsed in buffer, dehydrated through a graded ethanol series and embedded in Lowikryl K4M at -20ºC, as described by Carlemalm et al. (1982).

6.2.4.2. Immunocytochemical localisation

We used a polyclonal antibody prepared against spinach Rubisco raised in rabbits. The antibody was previously tested in wheat mediating western-blotting, and non-cross reactivity against other proteins was found (Dr. Guiamet, personal communication).

Immunocytochemical localisation was performed in cross-sections of the organs (*ca.* 1 μm). To block non-specific antibody sticking, first we incubated the cuts in 0.1 M phosphate-buffered saline (PBS) containing 1% (w/w) bovine serum albumin (BSA) for 15 min. Then, the cuts were incubated for 60 min with a dilution 1:10 (v/v) of a polyclonal antibody against Rubisco detailed above. After this, antibody solution was removed by washing the cuts with PBS-BSA several times. The cuts were incubated with a dilution 1:85 (v/v) of protein A-gold (5 nm) in PBS-BSA for 120 min. Non-specifically bound gold was removed by washing several times in PBS and finally with water. Controls included omission of the primary antiserum. Silver intensification was performed for 15 min with a Silver Enhancing Kit (British Bio-Cell International, UK) following the instructions of the manufacturer. The cuts were then immediately washed with water. The cuts were stained with methylene blue and photographed with light (reflected) microscopy (Olympus BHZ-UMA). Images were taken with a JVC TK 1270 camera.

6.3. Results

6.3.1. Plant water status and osmotic adjustment

In the greenhouse experiment, flag leaves of water-stressed (*WS*) plants showed a steady and significant ($P < 0.05$) decrease in their ψ_w and *RWC* (Table 1). In well-watered (*WW*) plants, *RWC* and ψ_w did not significantly change throughout the experiment. In the field experiment, significant differences in ψ_w and *RWC* between water regimes were also observed (Table 1).

Table 1. *Water potential (Ψ_w) and relative water content (RWC) of the flag leaves in well-watered (WW) and water-stressed (WS) plants of durum wheat (Triticum turgidum L. var. durum) grown under both greenhouse and field conditions. In the greenhouse, measurements were made at anthesis, and 15 and 30 days after anthesis. In the field, measurements were performed 25 days after anthesis. Values are the mean of four to six measurements ± s.e. Significant differences ($P \leq 0.05$) between well-watered and water-stressed plants according to the LSD test are denoted with an asterisk.*

	Treatment	**Ψ_w** (MPa)	**RWC** (%)
Greenhouse			
Anthesis	***WW***	-1.07 ± 0.09	90.26 ± 1.63
	WS	-1.64 ± 0.19 *	80.91 ± 5.41 *
15 days after anthesis	***WW***	-1.01 ± 0.09	88.45 ± 1.17
	WS	-1.86 ± 0.17 *	77.34 ± 2.75 *
30 days after anthesis	***WW***	-1.15 ± 0.08	85.29 ± 1.50
	WS	-2.33 ± 0.15 *	68.06 ± 3.27 *
Field			
25 days after anthesis	***WW***	-1.71 ± 0.24	85.98 ± 2.46
	WS	-2.37 ± 0.23 *	67.49 ± 5.25 *

The *RWC* of all the (non-senescent) leaves of the culm plus various ear parts of plants grown in the greenhouse was analysed 15 days after anthesis. The *RWC* showed a bottom-to-top gradient in *WS* plants, with the higher value in the awns (Figure 1A). In the *WW* plants, no

relevant differences were observed in *RWC* of the leaves and ear parts (Figure 1A). However, when we contrasted the *RWC* of several organs (lower and flag leaves, glumes, lemmas and awns) in *WS* plants versus the water content (expressed as percentage of fresh weight) of the same organ in *WW* plants, a strong negative correlation ($r^2 = 0.93$) was found (Figure 1B). The awns showed the lowest water content under well-watered conditions and, as mentioned above, higher *RWC* in water-stressed plants.

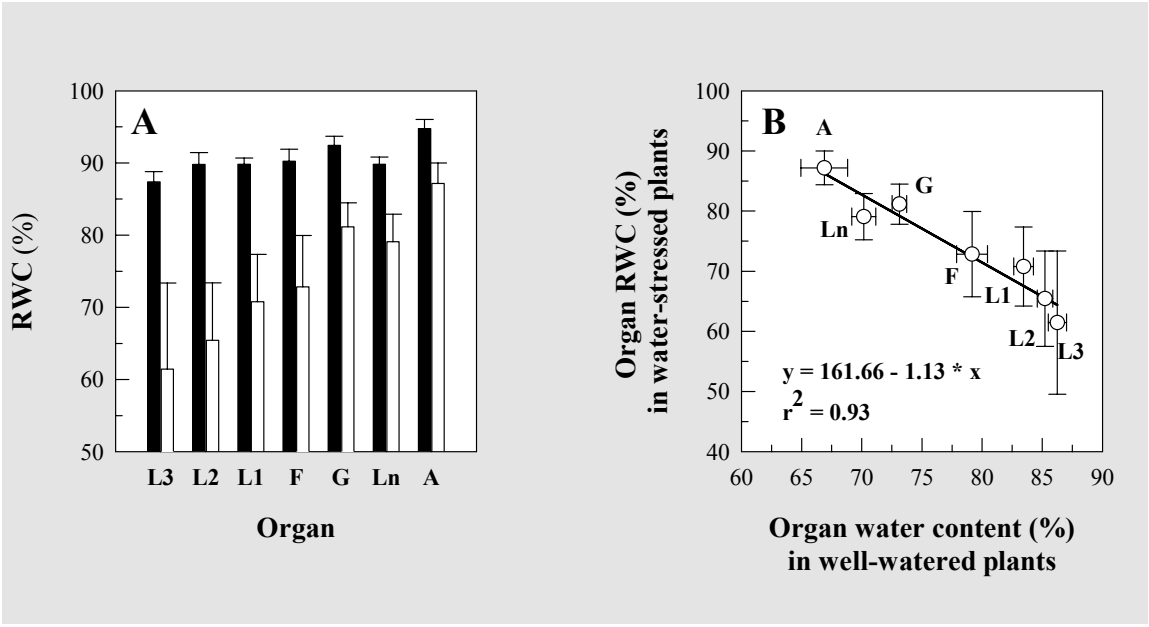


Figure 1. *Panel A. Relative water content (RWC) of leaves and ear parts of well-watered (filled bars) and water-stressed (open bars) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. F, G, Ln and A correspond to the flag leaf, glume, lemma and awn, respectively. L1, L2 and L3 are the three leaves following the flag leaf, in basal direction. Each value represents the mean ± s.e. of four measurements. Panel B. Relationship between the water content (as % of fresh weight) of the leaves and ear parts in well-watered plants versus the RWC of the same organs in water-stressed plants. Each point represents the mean ± s.e. of four measurements. Abbreviations as in panel A. For both panels, measurements were performed 15 days after anthesis.*

Osmotic potential at full turgor of flag leaves and ear parts showed significant differences ($P < 0.05$) between *WW* and *WS* plants (Table 2). In the flag leaf, the osmotic adjustment (the difference in osmotic potential between *WW* and *WS* plants) was low (6%), but in the ear parts osmotic adjustment was about 40, 46 and 28% for glumes, lemmas and awns, respectively.

Table 2. *Osmotic potential of flag leaf and ear parts (glume, lemma and awns) of well-watered (WW) and water-stressed (WS) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. Osmotic potential (MPa) was determined at full turgor by an osmometer 20 days after anthesis. The percentage difference in osmotic potential between WS and WW plants (i.e. osmotic adjustment) is also indicated. Each value represents the mean ± s.e. of four to six measurements. Significant differences ($P \leq 0.05$) between well-watered and water-stressed plants according to the LSD test are denoted by an asterisk.*

	Osmotic potential at full turgor (MPa)		
Organ	**WW**	**WS**	**Difference (%)**
Flag leaf	-1.43 ± 0.01	-1.52 ± 0.03 *	6.3
Glumes	-1.09 ± 0.08	-1.53 ± 0.02 *	40.4
Lemmas	-1.04 ± 0.07	-1.52 ± 0.02 *	46.1
Awns	-1.54 ± 0.10	-1.97 ± 0.05 *	27.9

6.3.2. Photosynthetic performance during grain filling

6.3.2.1. Greenhouse experiment

Photosynthetic response to water stress of the flag leaf and the ear was measured at three moments during grain filling. In *WW* plants, the light-saturated rate of CO_2 assimilation (A_{sat}) slightly decreased in the flag leaf and the ear, mainly between anthesis and two weeks later (Figure 2A). In addition, water stress led to a significant decrease (compared with *WW* plants) of A_{sat} in the flag leaf at the three moments of measurement (Figure 2A). In the ear, this decrease of A_{sat} by water stress was only significant ($P < 0.05$) at mid-grain filling (data not shown). Since the A_{sat} of the ear could be strongly influenced by the CO_2 emitted by this dark respiration (grain plus heterotrophic tissues), we also calculated the corrected A^*_{sat}, as $A_{sat} + R_d$ (Araus et al., 1993a). We observed no significant differences ($P < 0.05$) in the A^*_{sat} of the ear between *WW* and *WS* plants (Figure 2B). The dark respiration rate (R_d) in the ear showed a

peak 15 days after anthesis in both *WW* and *WS* plants (Figure 2C). At 30 days after anthesis, the R_d was similar to anthesis (Figure 2C).

Modulated chlorophyll fluorescence measurements showed steady decay of ϕ_{PSII} in the flag leaves of *WS* plants, mainly 30 days after anthesis (Figure 3A). This decrease of ϕ_{PSII} was accompanied by decreases in both photochemical quenching (*qP*; Figure 3B) and in the F_v'/F_m', the efficiency of energy capture by open *PSII* reaction centres (Figure 3C). In contrast, no significant ($P < 0.05$) differences between *WW* and *WS* plants were observed in fluorescence parameters of the ear (Figure 3 A, B and C; right panels). Nor did water stress lead to any significant effect on the potential yield of *PSII* (i.e. F_v/F_m in dark-adapted leaves) in the two organs (data not shown).

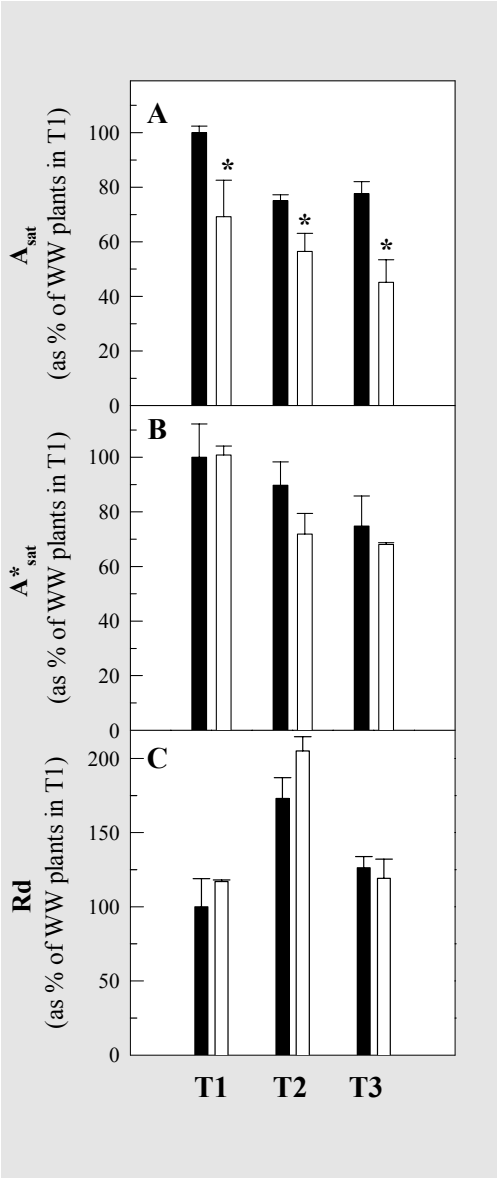


Figure 2. *Changes during grain filling in the light-saturated net photosynthetic rate (A_{sat}) of the flag leaf (**A**), and the corrected photosynthetic rate ($A^*_{sat} = A_{sat}$ + Rd) and dark respiration (Rd) of the ear (**B, C**) of well-watered (filled bars) and water-stressed (open bars) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. The measurements were performed at anthesis, and 15 or 30 days after anthesis (T1, T2 and T3, respectively). Values are expressed as percentage of the measurements at anthesis (T1) under well-watered conditions. Each value represents the mean ± s.e. of four to six measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted by an asterisk.*

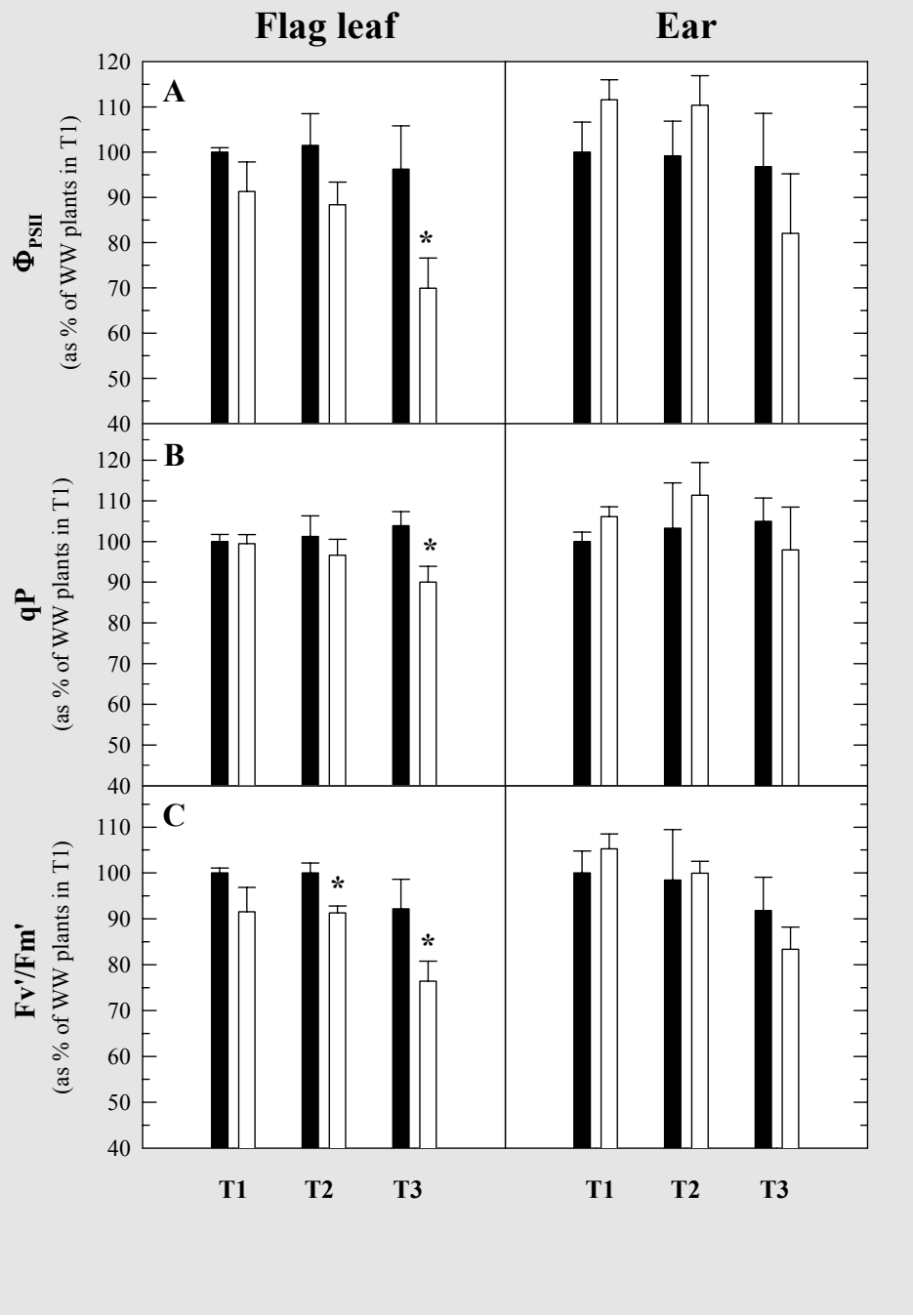


***Figure 3**. Changes in effective quantum efficiency of PSII (ϕ_{PSII}, **A**), photochemical quenching (qP, **B**) and efficiency of energy capture by open PSII reaction centres (F_v'/F_m', C) of flag leaves (left side of figure) and ears (right side) of well-watered (filled bars) and water-stressed (open bars) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. The measurements were performed at anthesis, and 15 or 30 days after anthesis (T1, T2 and T3, respectively). Values are expressed as percentage of the measurements at anthesis (T1) under well-watered conditions. Each value represents the mean ± s.e. of four to six measurements. Significant differences ($P \leq 0.05$) between treatments according to the LSD test are denoted by an asterisk.*

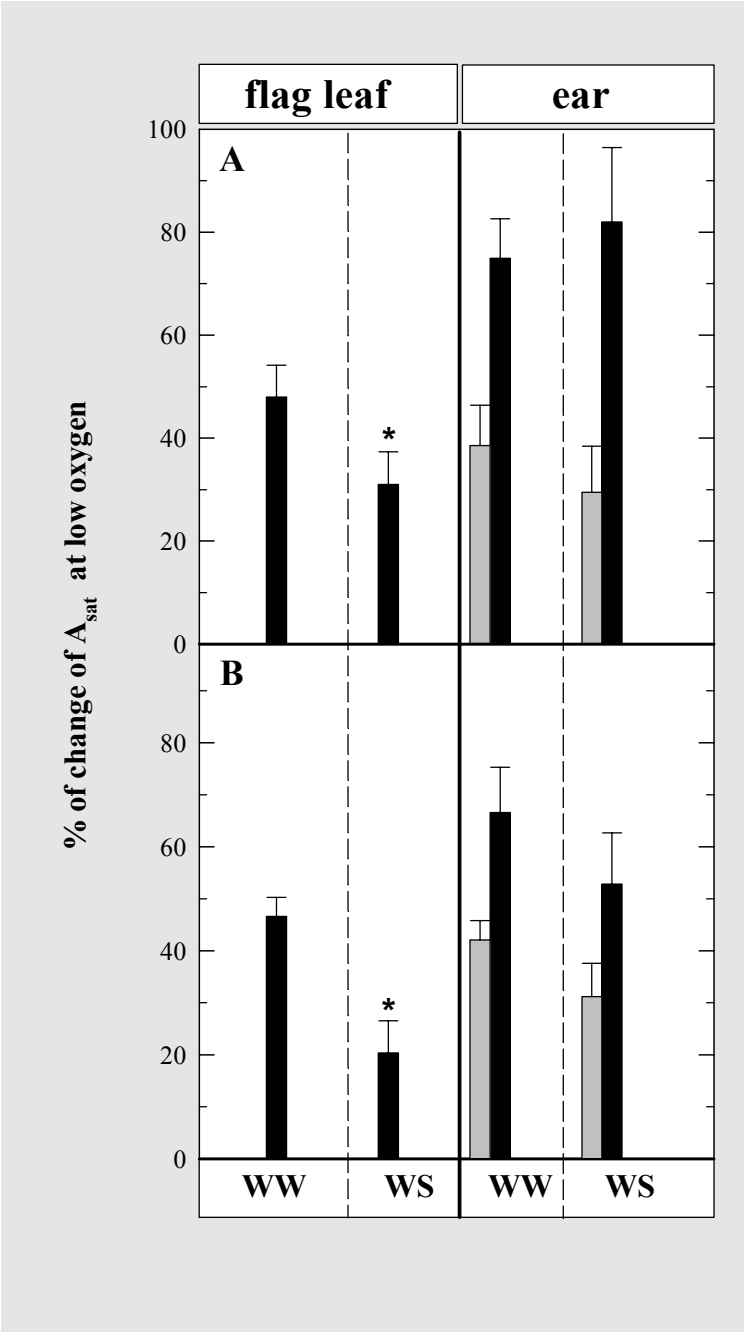


***Figure 4**. Low-oxygen sensitivity of the light-saturated net photosynthetic rate (A_{sat}) of well-watered (WW) and water-stressed (WS) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. Measurements were taken 15 days (**A**) and 30 days (**B**) after anthesis. The results (filled bars) are expressed as the percentage of change of the A_{sat} at low (ca. 2%) oxygen versus normal atmospheric (ca. 21%) oxygen. In the case of the ear, the response of the A^*_{sat} (A_{sat} + R_d) was also calculated in a similar way (grey bars). Each value represents the mean ± s.e. of four to six measurements. Significant differences ($P \leq 0.05$) between treatments (WW versus WS) according to the LSD test are denoted by an asterisk.*

6.3.2.2. Field experiment

The field photosynthetic responses of the flag leaf and the ear to water stress were similar to the greenhouse ones. Thus, about 4 weeks after anthesis, as response to water stress, the ear showed less decrease in A_{sat} and ϕ_{PSII} than the flag leaf (Table 3).

Table 3. *Comparison of decreases in the photosynthetic rate (A_{sat}) and relative quantum yield of PSII photochemistry (ϕ_{PSII}) of flag leaves and ears in water-stressed and well-watered plants of durum wheat (Triticum turgidum L. var. durum) growing in greenhouse and field conditions. Gas exchange and fluorescence were measured simultaneously at 1000 μmol m^{-2} s^{-1} of PPFD and 25ºC, 30 days (greenhouse) and 25 days (field) after anthesis. The decrease of A^*_{sat} (i.e. A_{sat} + R_d) for ears is also shown (in brackets). Values are expressed as the percentage decrease of WS compared with WW plants.*

	Greenhouse (T3)		**Field**	
	A_{sat} (%)	ϕ_{PSII} (%)	A_{sat} (%)	ϕ_{PSII} (%)
Flag leaf	42.8	27.8	56.3	24.3
Ear	9.8 (9.0)	10.3	25.0 (5.3)	17.1

6.3.3. Photosynthetic response to low oxygen

The photosynthetic response to low oxygen was measured in *WW* and *WS* plants 15 and 30 days after anthesis in the greenhouse experiment (Figure 4). In the flag leaf, the increase of A_{sat} at low O_2 was *ca.* 45% in *WW* plants. However, this stimulation of A_{sat} was lower in flag leaves in *WS* treatment, and decreased as water stress developed during grain filling (Figure 4A**,** B; filled bars in left panels). In the ear, A_{sat} increased markedly (*ca.* 80% and 60% 15 and 30 days after anthesis, respectively) at low oxygen (Figure 4A, B, filled bars in right panels). However, for the corrected photosynthetic rate (A^*_{sat}), an increase similar to that in the flag leaf was found (Figure 4A, B, grey bars). As response to water stress, no significant decrease in the stimulation of A^*_{sat} was shown.

The ϕ_{PSII} response curves to CO_2 under photorespiratory and non-photorespiratory conditions were also analysed in *WW* and *WS* ears and flag leaves at mid-grain filling (Figure 5). At normal atmospheric O_2 concentration, and regardless of the water treatment, both flag

leaf and ear showed moderate decrease of electron transport at lower CO_2 concentrations (Figure 5A). Under non-photorespiratory conditions, this decrease was greater (compared with normal oxygen) in the two organs of *WW* and *WS* plants (Figure 5B).

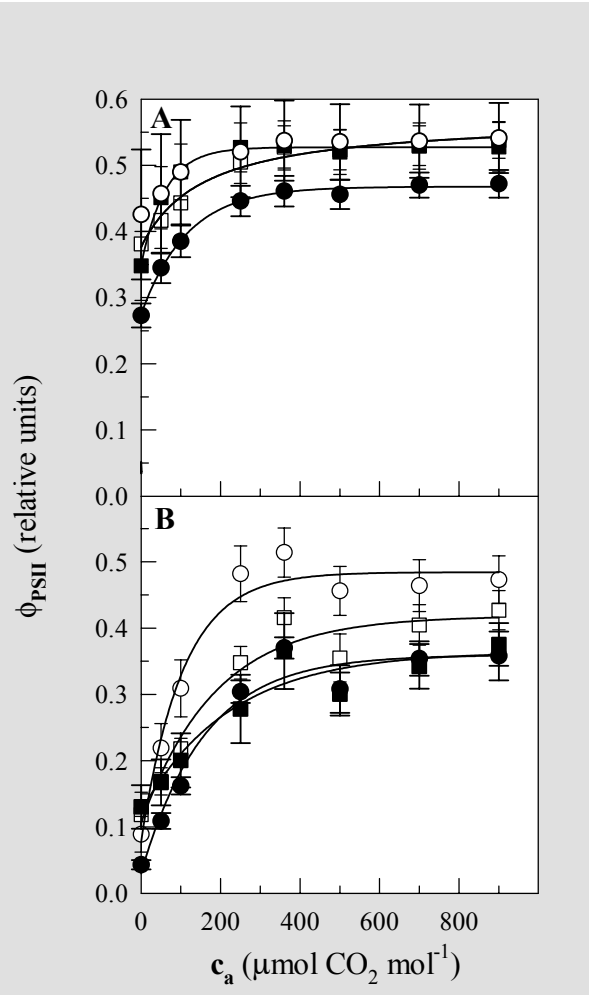


Figure 5. *Response of relative quantum yield of PSII photochemistry (ϕ_{PSII}) to CO_2 changes at normal (ca. 21%) (A) or low (ca. 2%) (B) oxygen concentration of flag leaves (circles) and ears (squares) of well-watered (open symbols) or water-stressed (closed symbols) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. The measurements were taken 15 days after anthesis. The curves were performed at 800 µmol m^{-2} s^{-1} of PPFD. Each value represents the mean ± s.e. of four to six measurements.*

6.3.4. Anatomy and Rubisco distribution

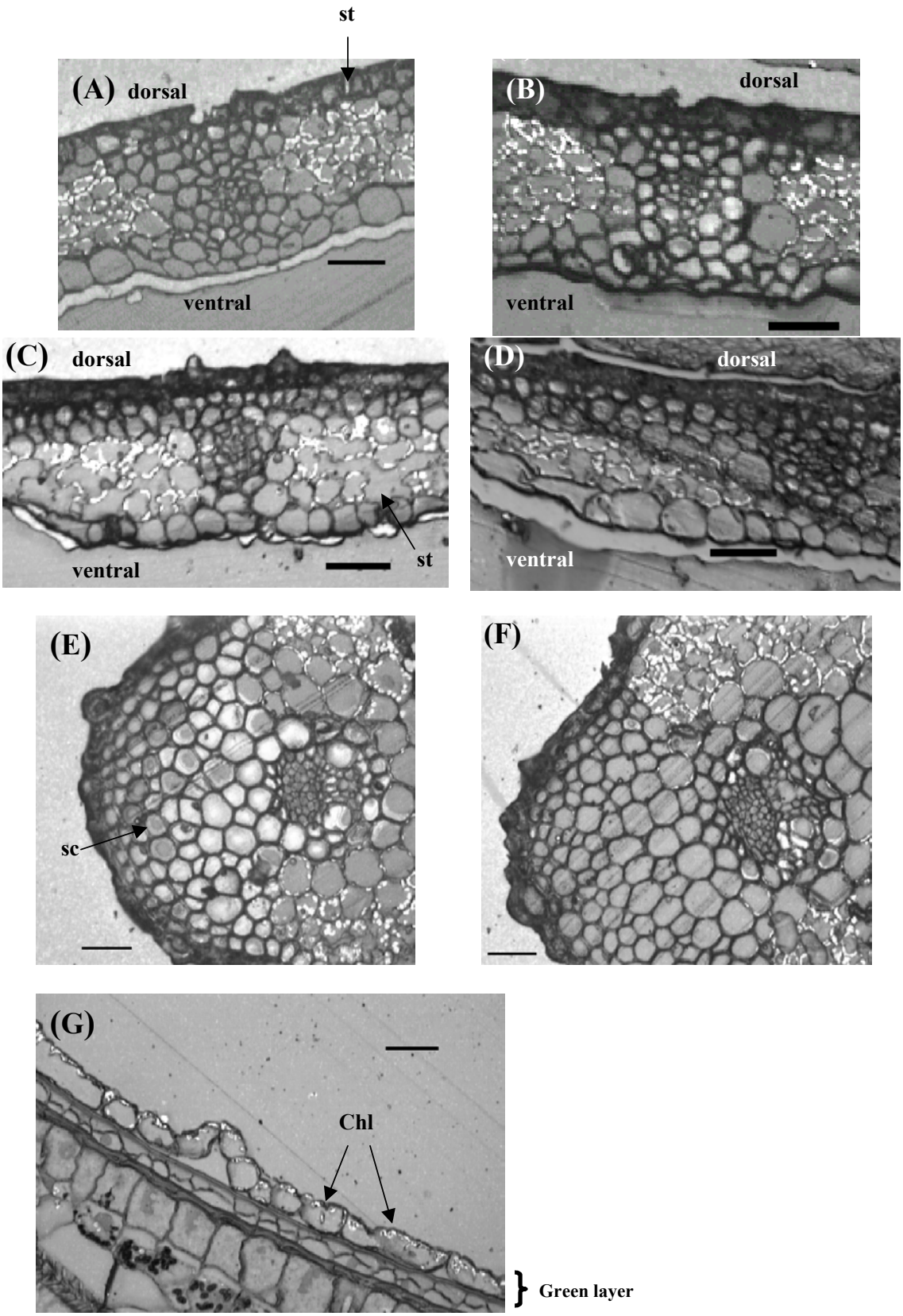
Ear parts had typical non-Kranz anatomy. Thus, as well as the lack of a developed bundle sheath with chloroplast, in the glumes mesophyll cells were positioned both on the dorsal and ventral sides (Figure 6 A,B), while in the lemma (at least in the area superimposed on the glume) the photosynthetic cells were distributed on the ventral side (Figure 6 C,D).

The immunolocalization of Rubisco analysed by means of optical microscopy did not support any compartmentalisation in the distribution of this protein in ear parts (Figure 6). In

fact, silver label (viewed as brilliant areas in the chloroplasts) was uniformly distributed in the mesophyll cells of glumes, lemmas and awns, both in *WW* and *WS* plants. In general, the label was absent from other compartments such as the cells surrounding the vessels. We also analysed the Rubisco distribution in the green layer of the pericarp of the grain. In this tissue, chloroplasts are distributed on the tangential side of the cells and were labelled against Rubisco antibody (Figure 6G).

Microscopic observation showed the xeromorphic nature of bracts and awns, with the glume and the lemma having a dorsal epidermis and sub-epidermal sclerenchymatous tissue with thick walls (see Figure 6 A,C). In contrast, the ventral epidermis (facing the grain) of both organs had cells with thin walls.

Figure 6 (página siguiente). *Light micrographs of Rubisco immunolocalization in cross-sections of glumes (A, B), lemmas (C, D) and awns (E, F) of well-watered (left) and water-stressed (right) plants of durum wheat (Triticum turgidum L. var. durum) grown in a greenhouse. Immunolocalization of Rubisco in cross-section of green grain pericarp in WS plants is also shown (G). In the lemma the cross-sections correspond to the area superimposed on the glume (i.e. central area). Semi-thin sections were treated with specific polyclonal anti-Rubisco antibody. Immunolabelling was performed by Protein A-gold and silver intensification. Label of Rubisco is visualised as brilliant areas in the chloroplasts. chl, labelled chloroplasts in green layer; sc: sclerenchyma; st, stomata. bar = 40 μm.*


st
(A) dorsal
ventral
(B)
dorsal
ventral
(C) dorsal
ventral
st
(D)
dorsal
ventral
(E)
sc
(F)
(G)
Chl
Green layer

6.4. Discussion

6.4.1. C_3 versus C_4 metabolism in the ear

The possibility that there is some degree of C_4 metabolism in the ear of C_3 cereals, such as wheat, barley and rice, has been reported (or at least suggested) in several studies (Nutbeam and Duffus 1976; Wirth et al. 1976; Singal et al. 1986; Ziegler-Jöns 1989; Imaizumi et al. 1990). However, in the present study, ear and flag leaf photosynthesis showed a typical C_3-response to low oxygen (*i.e.* non-photorespiratory) conditions in *WW* plants (Figure 4). Thus the photosynthetic rate of both organs was stimulated by low oxygen at *ca.* 45%. In the flag leaf under water stress, oxygen sensitivity was lower than in *WW* plants, whereas in the ear the decrease was not significant. A decrease in low-oxygen stimulation of A_{sat} as response to water stress has been attributed to phosphate limitation (Sharkey 1985; Nogués and Baker 2000; Nogués and Alegre 2002). Inorganic phosphate (P_i), essential for photophosphorylation in the stroma, can be limiting if phosphorylated intermediates are not exported from the chloroplasts. In fact, O_2 insensitivity is considered a symptom of P_i deficiency *in vivo* in C_3 plants (Leegood 1989) and has been reported in water-stressed leaves (Sharkey 1985).

Therefore, the oxygen sensitivity of the photosynthetic rate in the ear was typical of C_3 plants, and was not significantly affected by water stress (Figure 4). This observation is consistent with some previous reports, in which no C_4 metabolism was found in the ear of bread wheat (Bort et al. 1995). However, whereas in this latter study only well-watered plants were analysed, our results do not support an induction of C_4 metabolism in the ears as response to water stress.

A similar conclusion can be drawn from the analysis of the ϕ_{PSII} response to decreasing CO_2 at low oxygen (Figure 5). Decreasing the concentration of CO_2 in non-photorespiratory conditions caused the ϕ_{PSII} in the ear (as in the flag leaf) to drop markedly. This CO_2-O_2 interaction is interpreted as strong evidence for Rubisco-mediated carbon assimilation as a sink of electron transport (Lawson et al. 2002). This is further clear evidence for C_3 metabolism in the ear, which has not previously been reported.

Nor does the lack of Kranz anatomy (Figure 6) support the existence of C_4 metabolism in the ear of durum wheat. Ziegler-Jöns (1989) reported an anatomy-like intermediate C_3-C_4 in bracts (glumes and lemmas) of bread wheat. However, the evidence for this was very indirect, and compartmentalisation of enzymes such as Rubisco or PEP carboxylase (*PEPc*) was not

analysed. Recently it has been demonstrated that Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis (Voznesenskaya et al. 2001; Sage 2002). These authors showed that the spatial separation of the biochemical events necessary for C_4 photosynthesis occurs within a single cell in some halophyte plants. However, we did not observe any compartmentalisation in Rubisco protein, at either the histological or cellular level (Figure 6). Thus, the evidence shown in this paper does not support the existence of C_4 metabolism in the ear of durum wheat.

Some studies report the existence in the ear of enzymes of the C_4 metabolism, such as *PEPc* (Wirth et al. 1976) and pyruvate orthophosphate dikinase (*PPDK*) (Aoyagi and Bassham 1984). However, the interpretation of their findings is not straightforward. *PEPc* is a ubiquitous enzyme in vegetal tissues and is non-exclusive of C_4 metabolism (Chollet et al. 1996). In fact, immunocytochemical localisation of *PEPc* suggests no existence of C_4 metabolism in the ear, at least in glumes of durum wheat (Araus et al. 1993b). Furthermore, it is known that *PPDK* is present in C_3 tissues (Aoyagi and Bassham 1984). As indicated by Araus et al. (1993b), the function of these enzymes seems to involve anaplerotic rather than photosynthetic CO_2 fixation. In short, these enzymes are unlikely to be involved in photosynthesis, which is congruent with our results. Other studies giving evidence of C_4 metabolism can be reinterpreted. For instance, O_2 insensitivity reported in spikelets of rice (Imaizumi et al. 1990) could be explained in other ways (for example, phosphate limitation), as discussed above. In addition, the carbon isotope composition of bracts and awns is higher than that of the flag leaf (Araus et al. 1992a,b; Araus et al. 1993a; Gebbing and Schnyder 2001), but their value (ranging between *ca*. -22 and -30‰) seems to fall within the normal range of C_3 species (Pate 2001).

It is worth noting that, since gas exchange measurements only analyse the assimilation of external CO_2, we could not discard the existence of some grade of C_4 metabolism in the refixation of respired CO_2 (Bort et al. 1996; Gebbing and Schnyder 2001). However, as mentioned above, the electron transport of the ear parts studied is clearly sensitive to oxygen and CO_2 in a similar way to the flag leaf (Figure 5B), suggesting that, despite the CO_2 source, ear photosynthesis is mainly C_3 type. In this respect, the mesophyll cells of the lemmas facing the grain (*i.e.* on the ventral side), which are probably involved in refixation of respired-CO_2 (Araus et al. 1993a), show a distribution of Rubisco typical of C_3 plants (Figure 6 C, D).

Though the green pericarp of developing grains (Caley et al. 1990) was not examined in our study, its relevance as a photosynthetic part of the ear seems minor (Figure 6 G).

6.4.2. Photosynthetic performance of ear *versus* flag leaf under water stress

The photosynthetic rate and the ϕ_{PSII}, F_v'/F_m' and q_P parameters of the ear were less affected by water stress than the flag leaf under both greenhouse (Figures 2 and 3) and field (Table 3) conditions. The better photosynthetic performance of the ear (compared with the flag leaf) under water-stress conditions has been previously reported in some studies (Johnson et al. 1974; Xu et al. 1990), although the mechanistic basis is lacking. The greater tolerance of ear photosynthesis to water stress could be explained by the possibility of intrinsic or water-stress-induced C_4 photosynthesis, which is not supported by our results. Alternatively, the existence of traits associated with drought tolerance, such as xerophytic structure, is discussed below.

The better photosynthetic performance of the ear than the flag leaf under water-stress conditions seems connected to its capacity to maintain higher *RWC* (Figure 1). Xu and Ishii (1990) also concluded that the capacity of bread wheat (*Triticum aestivum* L.) to maintain high *RWC* seemed to be the main cause of higher drought resistance in the ear. However, these authors measured the *RWC* of the whole ear (so including the growing grains) instead of the *RWC* of the different parts of the ear (*i.e.* glumes, lemmas and awns) separately. Consequently, their *RWC* values are strongly influenced by the high water content of the grains (Xu and Ishii 1990). Moreover, these authors reported for glumes similar water relations as for the flag leaf and suggested that the grain is responsible for ear tolerance to water stress. We disagreed with these authors, for we found differences in *RWC* between the flag leaf and ear bracts (and, even more, the awns) of *WS* plants (Figure 1). Recently, Wardlaw (2002) reported that the glumes of bread wheat maintained higher *RWC* than the flag leaf under progressive water stress. Although this study did not analyse the photosynthetic performance of the ear, its data are consistent with our results.

Greater capacity for osmotic adjustment in the different ear parts than in the flag leaf (Table 2) may contribute to the higher *RWC* status (Table 1; Figure 1A) and the better photosynthetic performance (Figures 2 and 3) of the ear. These results corroborate those of Morgan (1980), who reports higher osmotic adjustment in ears of *Triticum* species. Osmotic

adjustment can ensure maintenance of turgor and gas exchange under water stress (Clarke 1987; Kikuta and Richter 1986; Serraj and Sinclair 2002). Although some controversy has arisen on the true role of osmotic adjustment performing drought adaptation in agronomic yield (Serraj and Sinclair 2002), in several reports osmotic adjustment has been noted as a crucial factor for drought tolerance in wheat (Morgan 1984; Morgan and Condon 1986; Sen Gupta and Berkowitz 1987; Ludlow et al. 1990; Blum et al. 1999).

In addition to osmotic adjustment, other factors could be involved in the drought response of ear photosynthesis. Vertical heterogeneity in sclerophyllous characteristics of wheat leaves (such as lower intercellular spaces, smaller and packed cells, thicker cellular walls and higher proportion of sclerenchymatous tissue) has been reported (Araus et al. 1986). In this sense, the vertical gradient of water content in several organs of *WW* plants, which correlated closely – and negatively - with the *RWC* of the same organs in *WS* plants (Figure 1B), could reflect this xeromorphic tendency in upper levels of the plant and thus tolerance to water stress. Consequently, the greater capacity of the ear to maintain higher *RWC* than the flag leaf could also be related to sclerophyllous traits of bracts and, in particular, of awns (see Figure 6).

We concluded that the better photosynthetic performance under water stress of the ear than the flag leaf of durum wheat is not associated with the existence of C_4 photosynthesis induction in the ear. Rather, it seems related to the maintenance of better water status in the ear than in the flag leaf, which is at least partially explained by higher osmotic adjustment combined with a more xerophytic structure.

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