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Photoinhibition in chilling stressed wheat and maize

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Abstract

At chilling stress, the contents of photosynthetic pigments decreased significantly in maize, but in wheat the contents of chlorophyll (Chl) remained unchanged whereas the contents of total carotenoids (Car) increased. In both species the contents of α+β carotene and lutein + lutein-5,6-epoxide remained unaffected, but the de-epoxidation state involving the components of the xanthophyll cycle increased. Under chilling stress the photosynthetic electron transport also displayed a general failure in maize but in wheat only photosystem (PS) 2 coupled to the water oxidation complex was inhibited. Moreover, in stressed maize the quinone pool decreased, while the low and high potential forms of cytochrome b_{559} increased. In wheat only the contents of cytochrome b_{559LP} decreased. Peroxidation of acyl lipids in the chloroplast lamellae became more distinct in chilling stressed maize but could also be detected in wheat. Thus in chilling stressed maize prevails an impairment of the acceptor site of PS2 while in wheat photodamage is restricted to the electron donation pathway from water to P680 or to the oxygen evolving complex.

Additional key words: carotenoids; chlorophyll; cytochrome b_{559}; lipid peroxidation; photosystems 1 and 2; proteins; quinones; *Triticum*: xanthophyll cycle; *Zea*.

Introduction

Controlled environment experiments on a wide range of species have demonstrated that exposure of leaves to sub-optimal growth temperatures in the presence of high, and sometimes moderate, irradiances can result in photo-inhibition, depressing photosynthesis (Krause 1994). Such depressions of photosynthetic performance arise when the rate of transfer of excitation energy from the antennae to the photochemical reaction centres exceeds the rate of transfer from these reaction centres to electron transport chain (Young and Britton 1990). Although PS1 operates at the same rate as PS2 and transforms an equivalent amount of energy, it is relatively immune to photodamage (Inoue *et al.* 1989, Gong *et al.* 1993). Moreover, the primary target of damage is PS2 (Lidon and Henriques 1993a, Krause 1994, Ramalho *et al.* 1997), and two distinct photoinhibitory mechanisms exist at the acceptor and donor sides (Barber and Andersson 1992). Both these mechanisms may operate *in vivo*, depending on the circumstances, and lead to inhibition of electron transport. The lability of PS2 is probably due to its chemical reactions, which include the creation of strong oxidants required for removing electrons from water and the complex chemistry of plastoquinone reduction, which is strongly influenced by protons.

Maize and wheat have C_4 and C_3 metabolisms, respectively. These plant species need similar growth temperatures (14-24 °C), but their sensitivity to chilling stress is different. The main objective of this study was to compare and identify under chilling conditions the main targets of photoinhibition in these two species.

Materials and methods

Plants: Wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) seeds were washed in distilled water and sterilised for 2 min by immersion in a 0.1 % $HgCl_2$ solution. The seeds were then washed 5 times in de-ionised water, placed in an oven at 28 °C for 24 h, and afterwards germinated for 3 d on moist filter paper at 28 °C. The seedlings

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were grown for two weeks in a glasshouse under a maximum natural daylight irradiance of 1 200-1 600 μmol m^{-2} s^{-1}, 21/27-13/16 °C day/night temperature, 14/10 h light/dark cycle, and relative humidity between 65 and 70 % in pots filled with a mixture of vermiculite (*Eurover-Vermiculita, Art. A*, Spain) and *Trio-hum* (*Klasmann-Deilmann* traysubstrat, Art. Nr. *4240*, Germany) tray substrate (2 : 3), being irrigated with Hoagland nutrient solution. To induce the chilling stress, the plants were transferred to growth chambers (PPFD of 1 500-1 700 μmol m^{-2} s^{-1}, 14/10 h light/dark cycle, and 6/9-3/4 °C light/dark temperature) for 6 d.

Pigment analyses: Chl and Car were extracted with acetone and measured at 663.2, 646.8, and 470.0 nm according to Lichtenthaler (1987). Car were isolated and quantified by chromatography as described by Lidon and Henriques (1992a), using as mobile phase benzol, acetone, and chloroform (35.7/35.7/28.6, v/v). Measurement of α+β carotene, lutein-5,6-epoxide + lutein, violaxanthin + anteraxanthin, and zeaxanthin was carried out at 464, 446, 441, and 451 nm using as extinction coeficients 2 200, 2 540, 2 500, and 2 480, respectively. The de-epoxidation state (DEPS) involving the components of the xanthophyll cycle—violaxanthin, antheraxanthin, and zeaxanthin—was calculed according to Schindler *et al.* (1994).

Photosynthetic electron transport rates: Chloroplasts of leaf tissues were isolated according to Droppa *et al.* (1987), with minor modifications introduced by Lidon and Henriques (1992b). Leaves were homogenised in a medium containing 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$, 2 mM ascorbate, 0.4 % bovine serum albumin (BSA), and 50 mM MES (pH 6.4). After filtration through four layers of nylon, chloroplasts were sedimented by centrifugation at 2 000×*g* for 4 min. The chloroplasts were washed twice in the isolation medium and re-suspended in a medium containing 0.33 M sorbitol, 10 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$, 0.4 % BSA, and 50 mM HEPES (pH 7.5). Photosynthetic activities were measured using a Clark-type oxygen electrode (*Hansatech*, Kings Lynn, UK), following Lidon and Teixeira (2000), at 25 °C and photosynthetic photon flux density (PPFD) of *ca.* 1500 μmol m^{-2} s^{-1}, obtained from a Björkman lamp (*Hansatech*). For determination of PS2 activity including the water oxidation complex, 2,6-dichlorophenolindophenol was used as electron acceptor from the quinone pool. For measurement of PS2 activity without the water oxidation complex, 1,5-diphenyl-carbohydrazide was used. To measure the electron transport associated to PS1, reduced 2,6-dichlorophenolindophenol was used as electron donor to the cytochrome b_6/f complex, with methylviologen as electron acceptor. Sodium azide and 3-(3,4-dichlorophenyl)-1,1-dimethylurea were also applied to inhibit peroxidase activities and the electron transport before the plastoquinone, respectively.

Photosynthetic electron carriers: Chloroplast isolation for cytochrome determinations, using 15 g of leaf tissue, was carried out as described by Lidon and Henriques (1993b). The concentrations of high and low potential forms of cytochrome b_{559} were measured according to Houchins and Hind (1984). Values were obtained at 545 nm using isosbestic wavelengths of 528 and 568 nm. An extinction coefficient of 20 mM^{-1} cm^{-1} was used.

To determine quinones, subchloroplast fractions were prepared of 10-g leaf samples according to Droppa *et al.* (1987), with minor modifications described by Lidon and Henriques (1993b). The chloroplast quinone pool was measured according to Redfearn and Friend (1962), with minor modifications. To the chloroplast preparation (1 cm^3), 4 cm^3 of cold methanol (–20 °C) containing pyrogallol (13 g m^{-3}) was added. Light petroleum (6 cm^3) was then added and the mixture was shaken for 2 min. After centrifugation (2 min at 200×*g*), the light petroleum layer was removed and another extraction with light petroleum (4 cm^3) was made. The light petroleum extracts were combined, and 4 cm^3 of aqueous methanol (90 %) were added. The mixture was shaken and the layers were separated by centrifugation for 2 min at 100×*g*. Methanol layer was removed and the partitioning process with methanol (90 %) continued until this solution layer was colourless. The yellow, light petroleum layer that contained the quinone pool was evaporated in a vacuum desiccator and the residue was dissolved in ethanol (3 cm^3). The spectrum of ethanol solution was determined at 230 to 320 nm. The quinone pool was then reduced by the addition of 10 mm^3 of a sodium borohydride solution (600 g m^{-3}) followed by rapid stirring, and the spectrum was re-determined over the same range. Quinone concentration was calculated from the difference in extinction at 255 nm, using the molecular extinction coefficient of 14 800 mM^{-1} cm^{-1} (for the difference in absorption of the oxidised and reduced forms).

Thiobarbituric acid test and protein measurements: Leaf samples (*ca.* 2 g) were homogenised for 1 min with 0.4 M sucrose, 20 mM Tricine-KOH (pH 8.0), 10 mM sodium chloride, and 30 mM sodium ascorbate. After filtration, using four layers of cheesecloth, and centrifugation at 300×*g* (2 min, 4 °C), the supernatant was recovered and centrifuged again at 5 000×*g* (5 min, 4 °C). The sub-chloroplast fractions obtained in the pellet were mixed with 20 mM tricine-KOH (pH 8.0), 10 mM sodium chloride, and 30 mM sodium ascorbate and centrifuged at 5 000×*g* (10 min, 4 °C). The washed pellet was then re-suspended in 50 mM phosphate buffer (pH 7.5), and the thiobarbituric acid test, which determines malondialdehyde as an end product of lipid peroxidation, applied to determine the extent of peroxidation of acyl

lipids (Cakmak and Horst 1991). The subchloro-plast fraction (2 cm^3) was added to 5 cm^3 of 20 % trichloro-acetic acid and 1 % thiobarbituric acid, and incubated at 95 °C for 10 min. After cooling the reaction tubes in an ice-water bath, the samples were centrifuged at 3 000×*g* for 5 min, and the absorbance of the supernatant was read at 535 nm. The amount of malondialdehyde-thiobarbituric acid complex was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Protein concentration was measured according to Lowry *et al.* (1951) in chloroplast lamellae extracted according to Droppa *et al.* (1987). A BSA standard curve was used.

Statistical analysis was performed by two-way ANOVA (*F*-ratio test, for $p \leq 0.05$ multiple range analysis for a 95 % confidence level). Different characters indicate significant differences: *a* and *b* between control and chilling stressed plants, *r* and *s* between wheat and maize within each treatment. Each value was the mean of triplicates from five independent series.

Results

Under chilling stress, the concentrations of Chl *a* and *b* did not vary much in wheat, but in maize were severely depressed (to 28.4 and 48.1 %, respectively), the Chl *a/b* ratio being reduced by 30 % in wheat and by 59 % in maize (Table 1). The control plants of maize also showed higher contents of Chls than wheat, but under chilling stress an opposite trend was found. The conjunction of these patterns revealed a higher rate of pigment damage in maize. The content of total Car displayed opposite trends, achieving a significant 25.0 % increase in chilled wheat but a 43.8 % decrease in maize (Table 1). However, in the control and chilling stressed maize the contents of Car remained higher then in wheat, further expressing a higher ratio with Chl (Table 1). Under chilling stress the relative proportions between Car and Chls did not vary significantly in wheat but increased in maize (Table 1). In stressed wheat and maize the contents of α+β carotene and lutein + lutein-5,6-epoxide did not vary significantly, but the content of violaxanthin + antheraxanthin was depressed to 48.8 and 42.7 %, respectively, whereas zeaxanthin reached 1.43- and 1.68-fold increases, respectively (Table 2). In the de-epoxidation state involving the components of the xanthophyll cycle, 1.34- and 1.21-fold increases were found for wheat and maize, respectively (Table 2). In chilling-stressed maize the photosynthetic electron transport associated to PS1 and PS2 (with and without the water oxidation complex) was reduced to 37.8, 37.6, and 49.7 %, respectively (Fig. 1).

Table 1. Contents of chlorophylls (Chl) and carotenoids (Car) per fresh mass unit in chilling stressed and non-stressed wheat and maize plants. Each value is the mean of triplicates ± S.E.

Pigment	Wheat		Maize	
	Control	Chilling	Control	Chilling
Chl *a* [g kg^{-1}]	0.98 ± 0.04^{ar}	1.02 ± 0.01^{ar}	2.22 ± 0.01^{as}	0.63 ± 0.01^{bs}
Chl *b* [g kg^{-1}]b	0.35 ± 0.05^{ar}	0.52 ± 0.01^{ar}	0.79 ± 0.01^{as}	0.38 ± 0.01^{bs}
Chl (*a*+*b*) [g kg^{-1}]	1.30 ± 0.10^{ar}	1.54 ± 0.01^{ar}	3.01 ± 0.03^{as}	1.01 ± 0.01^{bs}
Chl *a/b*	2.80 ± 0.30^{ar}	1.96 ± 0.04^{ar}	2.80 ± 0.05^{ar}	1.65 ± 0.01^{bs}
Car [g kg^{-1}]	0.28 ± 0.01^{ar}	0.35 ± 0.00^{br}	0.96 ± 0.15^{as}	0.54 ± 0.00^{bs}
Car/Chl	0.21 ± 0.02^{ar}	0.22 ± 0.04^{ar}	0.32 ± 0.03^{ar}	0.54 ± 0.01^{bs}

Table 2. Relative proportions of carotenoids [%] and DEPS in chilling stressed and non-stressed wheat and maize plants. Each value is the mean of triplicates ± S.E.

Carotenoid	Wheat		Maize	
	Control	Chilling	Control	Chilling
α+β carotene	34.1 ± 2.0^{ar}	32.3 ± 2.0^{ar}	25.8 ± 1.0^{as}	23.4 ± 1.0^{as}
Lutein+lutein-5,6-epoxide	28.1 ± 0.5^{ar}	29.4 ± 0.5^{ar}	37.0 ± 3.0^{as}	38.1 ± 3.0^{as}
Violaxanthin+antheraxanthin	16.8 ± 0.5^{ar}	8.2 ± 0.5^{br}	19.2 ± 1.0^{ar}	8.2 ± 1.0^{br}
Zeaxanthin	21.0 ± 1.0^{ar}	30.1 ± 1.0^{br}	18.0 ± 1.0^{ar}	30.3 ± 1.0^{br}
DEPS	0.666^{ar}	0.893^{br}	0.742^{ar}	0.894^{br}

Moreover, in wheat these electron transport rates were inhibited in PS2 coupled to the water oxidation complex (decrease to 52.6 %), remained unchanged in the absence of O_2 evolution, and showed a 1.47-fold increase in PS1 (Fig. 1). The quinone pool only decreased in chilling stressed maize (to 62.5 %), whereas cytochrome b_{559HP}

showed a 1.22-fold increase in wheat (Fig. 1). In wheat the levels of cytochrome b_{559LP} decreased to 70 % but in maize showed a 1.46-fold increase (Fig. 1). Regardless to the degradation of chloroplast lamellae, peroxidation of acyl lipids became more distinct in maize but could also be detected in wheat (Table 3). The concomitant protein/Chl ratio was enhanced in maize but stayed rather unchanged in wheat (Table 3).

Table 3. Extent of lipid peroxidation [mmol(MDA) kg^{-1}(Chl)] and protein/chlorophyll ratio in the chloroplast lamellae of chilling stressed and non-stressed wheat and maize plants. Each value is the mean of triplicates ± S.E.

	Wheat		Maize	
	Control	Chilling	Control	Chilling
Lipid peroxidation)	100 ± 3^{ar}	130 ± 6^{ar}	$4\,700 \pm 100^{as}$	$7\,200 \pm 400^{bs}$
Protein/chlorophyll	14.4 ± 1.0^{ar}	15.2 ± 2.0^{ar}	4.67 ± 0.003^{as}	23.3 ± 0.5^{bs}

Discussion

If the photon amount is in excess of the photon-utilising capacity, excess photons produce reactive chemical entities in chloroplasts that can oxidise target molecules leading to photoinhibition. In our experiment, this physiological disorder was induced when wheat and maize were transferred from optimal growth temperatures to chilling stress at which the utilisation of excitation energy in carbon metabolism was reduced and thus the proportion of excess energy available to both photosystems at the given PPFD increased. The modifying action of chilling temperatures in conjunction with excess PPFD was, however, expressed by different patterns in these plants. The increased accumulation of total Car in the photosynthetic apparatus of wheat expanded the related excited state properties, limiting the destructive reactions of singlet oxygen *via* direct quenching of singlet oxygen or of the Chl triplet state that sensitises singlet oxygen formation (Cogdell and Frank 1987, Lidon and Henriques 1993a). Thus the Chl contents were maintained either because of the irreversible bleaching of these molecules, due to the loss of formation of singlet oxygen (Telfer *et al.* 1991), either because Chl triplets give away their spin to Car, preventing singlet oxygen formation (Lidon and Henriques 1993a). Moreover, the photochemical efficiency of wheat was also coupled to a higher rate of de-epoxidation state involving the components of xanthophyll cycle—violaxanthin, antheraxanthin, and zeaxanthin (for rice see Xu *et al.* 2000). The structural change that occurs on conversion of violaxanthin to zeaxanthin in the oxidation of epoxide groups forming cyclohexenyl ring increases the number of conjugated carbon-carbon double bounds. Switching the Car from being a donor of excitation to Chl to its being an acceptor (and quencher) of Chl singlet energy (Owens *et al.* 1992) influences the apparent polarity of the molecule as well as its ability to establish pigment-pigment interactions. In this context, the high conversion rate in wheat chloroplasts furnished an efficient photoprotection, minimising the yield of photo-generated reactive molecules mainly in the light-harvesting Chl protein 2 (Demmig-Adams *et al.* 1989, Lidon and Henriques 1993a). The pattern displayed by maize revealed a significant structural change of chloroplast lamellae, expressed by a general failure of accumulation of isoprenoids. The decreased content of total Car became a limiting factor, minimising the suppression of photo-produced reactive molecules, allowing additional photon energy transfer to

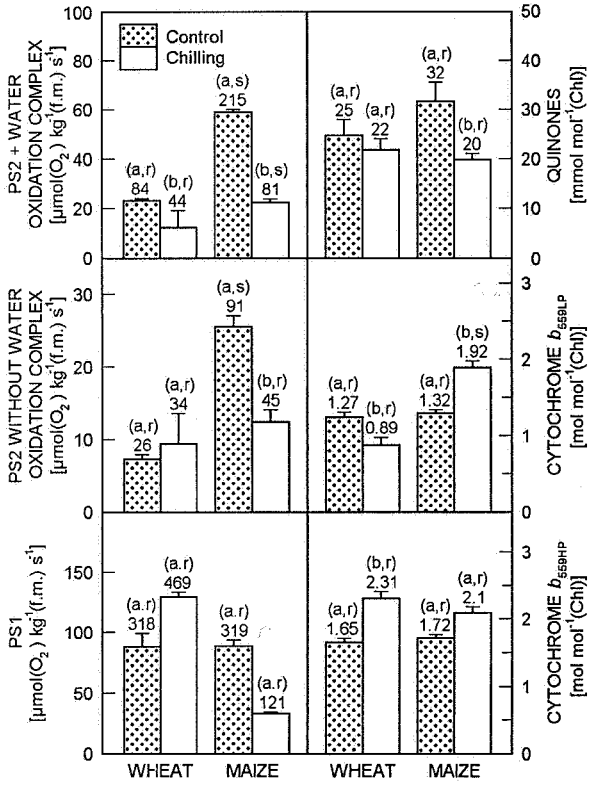

Fig. 1. Photosynthetic electron transport rates coupled to photosystem (PS) 1 and 2 (*left*) and photosynthetic electron carriers coupled to PS2 (*right*). In chilling-stressed maize an inhibition of the electron transport chain was found in multiple sites, but in wheat a restriction was detected only in PS2 coupled to the water oxidation complex.

Chl and dioxygen, and producing triplet excited Chl and singlet excited energy. These photo-damaging effects expressed through a sharp inhibition of Chl accumulation also point to an insufficient de-epoxidation or at least impairment in the xanthophyll cycle. In this context, the unchanged contents in maize and wheat of lutein + lutein-5,6-epoxide followed a tendency reported by Thayer and Björkman (1990) and Ramalho *et al.* (1997). This supports the findings of Siefermann-Harms (1985) and Schindler *et al.* (1994) that attribute to these pigments only an accessory role in the absorption and transfer of excitation energy to Chls within light-harvesting Chl-proteins. Moreover, in maize the impairment of photosynthetic electron transport associated to both photosystems was the most visible expression that the effective formation of zeaxanthin and energy-dependent quenching was strongly affected becoming insufficient at chilling temperatures (Demmig-Adams *et al.* 1989, Bilger and Björkman 1991). The general failure of light photosynthetic reactions of maize paralleled only a significant inhibition of the Hill reactions coupled to the water oxidation complex in wheat. Photo-damaging effects in PS1 of maize, though not as common as degradation of PS2, were reported in various plant species (Inoue *et al.* 1989, Gong *et al.* 1993). However, the photoinhibition found in PS2 of wheat and maize, although coupled to different inhibitory factors, seems to be the main target in multiple plant species (Krause 1994). In maize, the strong inhibition of the Hill reactions, even without the water oxidation complex, paralleled with a decreasing concentration of the quinone pool, suggesting that the acceptor side of PS2 involved high singlet oxygen synthesis implicating the formation of the P680 triplet state. This detrimental reaction probably occurred because the radical pair recombination was favoured due to the double reduction of quinone A (Vass *et al.* 1992) followed by its release from the binding site (Vass *et al.* 1988, Styring *et al.* 1990). The increasing content of the low potential form of cytochrome b_{559} also suggests the photoinhibition of the acceptor side of PS2 because the related electron potential allows its oxidation, thus being poised to act as an electron acceptor from reduced pheophytin. The maintenance of high contents of carotene found in chilling stressed maize (De Las Rivas *et al.* 1993) further suggests a protective role as an electron donor to PS2, limiting photo-damage at the donor side and improving the scavenging of singlet oxygen rather then by trapping Chl triplets. In wheat these mechanisms were, however, apparently surpassed. Indeed, in wheat the inhibition of Hill reactions could only be found when PS2 was coupled to the water oxidation complex and, furthermore, the content of the high potential form of cytochrome b_{559} increased. The PS2 cyclic electron transport involving cytochrome b_{559} is a mechanism that protects the reaction centre from damage due to the high irradiances (Canaani and Havaux 1990, Rees and Horton 1990, Buser *et al.* 1992) by reducing the damaging cation radicals (Buser *et al.* 1992). Thus, in wheat the photoinhibition in the chloroplast lamellae was apparently mostly restricted to the impairment of electron donation pathway from water to P680 (Erixon and Butler 1971, Lidon and Henriques 1993a) or to the direct degradation of the oxygen-evolving complex (Buser *et al.* 1990, Lidon and Henriques 1993a). Independent of the photo-damaging mechanisms triggered by chilling temperatures, the structure of thylakoid membranes of both plant species displayed significant degradation in acyl lipid bilayer, as seen by the synthesis of malondialdehyde-thiobarbituric acid complex, mostly due to the interaction of photo-generated oxy-radicals with unsaturated lipids promoting lipid hydroperoxide synthesis. However, the increasing ratio between protein and Chl found only in chilling stressed maize indicates a lower proteolysis.

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