

Photoinhibition in chilling stressed *Leguminosae*: comparison of *Vicia faba* and *Pisum sativum*

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Abstract

The concentrations of photosynthetic pigments decreased in both chilling stressed species but the ratios of chlorophyll (Chl) *a/b* and total carotenoids (Car)/Chls were depressed only in faba bean. The contents of $\alpha+\beta$ carotene and lutein+lutein-5,6-epoxide remained unaffected in both species, but the de-epoxidation state involving the components of xanthophyll cycle increased in pea. Under chilling stress the photosynthetic electron transport associated with photosystem 2, PS2 (with and without the water oxidising complex) decreased in both plant species, the inhibition being higher in faba bean. The intrachloroplast quinone pool also decreased in both stressed species, yet an opposite trend was found for cytochrome *b₅₅₉L_P*. Under stress an increasing peroxidation of thylakoid acyl lipids was detected in pea, but higher protein/Chl ratio was detected in faba bean. Thus the acceptor side of PS2 is mostly affected in both chilling stressed species, but faba bean is more sensitive.

Additional key words: carotenoids; chlorophylls; cytochromes; lipids; photoinhibition; photosystem 2; proteins; quinones; water oxidising complex; xanthophyll cycle.

Introduction

Controlled environment experiments with a wide range of plant species have demonstrated that exposure of leaves to sub-optimal growth temperatures in the presence of high, and sometimes even moderate, irradiances can result in photoinhibition, depressing photosynthesis (Krause 1994). The main site of photodamage is the photosystem 2 (PS2) reaction centre where rapid energy transformation occurs (Lidon and Henriques 1993b, Krause 1994, Ramalho *et al.* 1997). According to some authors, the damage begins on the reducing side of PS2. Other experiments, that often use material in which the water-oxidising system has been disconnected from primary photochemistry, show that the primary site of degradation is on the oxidising side of PS2 (Barber and Andersson 1992, Gong *et al.* 1993). To protect PS2 against photodegradation, the antenna system can divert exciton energy away from reaction centres (Demmig-Adams and Adams 1992). This mechanism involves Cars, namely those involved in the xanthophyll cycle. They interact with the antenna pigments in a manner that

converts exciton energy into heat, thereby decreasing the amount of energy reaching PS2 (Siefermann-Harms 1985, Bilger and Björkman 1991, De Las Rivas *et al.* 1993, Lidon and Henriques 1993b, Schindler *et al.* 1994, Lidon and Teixeira 2000). Additionally, within PS2 the electron transfer reactions implicating cytochrome *b₅₅₉* also play a central role by discharging potentially damaging radicals (Buser *et al.* 1990, 1992, Canaani and Havaux 1990, Rees and Horton 1990).

Faba bean and pea are two *Leguminosae* that need different temperatures for optimal vegetative growth. Temperatures between 7 and 24 °C are suitable for pea, but this species tolerates also lower temperatures. Moreover, between 6 and 8 °C vegetative growth stops in faba bean, for which the optimal temperature is between 12 and 20 °C. Following the different capabilities of faba bean and pea to cope with chilling stress, the main objective of this study was to compare and identify the main targets of photoinhibition.

Received 11 September 2000, accepted 8 November 2000.

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Acknowledgements: The authors thank Prof. Santos Oliveira (Grupo de Disciplinas de Ecologia da Hidrosfera, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Portugal) for laboratory facilities, Dr. José Ramalho (Instituto Investigação Científica Tropical – CEPTA, Portugal) for technical assistance in the growth chambers, and Tec. Eng. Carlos Carvalho for maintenance of plants in the glasshouse.

Materials and methods

Plants: Faba bean (*Vicia faba* L.) and pea (*Pisum sativum* L.) seeds were washed in distilled water and sterilised for 2 min by immersion in an 0.1 % mercury bi-chloride solution. Seeds were then washed 5 times in de-ionised water and placed for 24 h in an oven at 28 °C. The seeds germinated on moist filter paper at 28 °C for 3 d. The seedlings were grown in a glasshouse (under a maximum natural daylight irradiance of 1 200–1 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 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) for two weeks. They were irrigated with the Hoagland nutrient solution. To induce chilling stress, the plants were transferred to growth chambers (PPFD of 1500–1700 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 14/10 h light/dark cycle, and 6/9–3/4 °C light-dark temperature) for 6 d.

Pigment analysis: Chls *a* and *b* and Cars were extracted with acetone and measured at 663.2, 646.8, and 470.0 nm according to Lichtenthaler (1987). Isolation and quantification of Cars was carried out by chromatography as described by Lidon and Henriques (1992b), using as mobile phase benzol-acetone-chloroform (35.7 : 35.7 : 28.6, v/v/v). Determination of α + β -carotene, lutein-5,6-epoxide + lutein, violaxanthin + antheroxanthin and zeaxanthin was carried out 464, 446, 441, and 451 nm using extinction coefficients of 2 200, 2 540, 2 500, and 2 480, respectively. The de-epoxidation state (DEPS) involving the components of the xanthophyll cycle violaxanthin, antheroxanthin, and zeaxanthin was calculated according to Schindler *et al.* (1994).

Photosynthetic electron transport rates: Chloroplasts of leaf tissue were isolated according to Droppa *et al.* (1987), with minor modifications introduced by Lidon and Henriques (1992a). 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 % BSA, and 50 mM MES (pH 6.4). After filtration throughout four layers of nylon, chloroplasts were sedimented by centrifugation at 2 000 \times 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 % bovine serum albumin, and 50 mM HEPES (pH 7.5). Photosynthetic activities were measured in a Clark type oxygen electrode (*Hansatech*, Kings Lynn, U.K.), at 25 °C and PPFD of *ca.* 1 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, furnished by a Björkman lamp (*Hansatech*), following Lidon and Teixeira (2000). For PS2 including the water oxidising complex, 2,6-dichlorophenolindophenol was used as electron acceptor from the quinone pool. Without the water oxidation complex, 1,5-diphenyl-carbohydra-

zide was used as electron donor to PS2.

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

Sub-chloroplast fractions for determination of quinones were prepared according to Droppa *et al.* (1987) by using 10 g leaf samples, with minor modifications described by Lidon and Henriques (1993a). The chloroplast quinone pool was measured according to Redfean and Friend (1962), with minor modifications. To the chloroplast preparation (1 cm^3), 4 cm^3 of cold methanol (-20 °C) containing pyrogallol (1.3 kg 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 \times 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 \times 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 (60 kg 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 $\text{mM}^{-1} \text{cm}^{-1}$ (for the difference in absorption of the oxidised and reduced forms).

Thiobarbituric acid test and protein measurements: Leaf samples (*ca.* 2 g) were homogenised during 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 4 layers of cheesecloth and centrifugation at 300 \times g (2 min, 4 °C) the supernatant was recovered and centrifuged again at 5 000 \times 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 \times 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,

was applied to determine the extent of acyl lipid peroxidation, following Cakmak and Horst (1991). The sub-chloroplast fraction (2 cm^3) was added to 5 cm^3 of 20 % trichloroacetic 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 $3\,000\times g$ 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 $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Protein concentration was measured according to

Lowry *et al.* (1951) in chloroplast lamellae extracted according to Droppa *et al.* (1987). A bovine serum albumin standard curve was used.

Statistical analysis was performed by two way ANOVA (*F*-ratio test, for $p \leq 0.1$ multiple range analysis for a 90 % confidence level). Different letters indicate significant differences: *a* and *b* between control and chilling stressed plants; *r* and *s* between faba bean and pea within each treatment. Each value was the mean of triplicates from five independent series.

Results

Under chilling stress, the concentrations of Chl (*a+b*) were depressed in faba bean and pea to 79.3 and 65.2 %,

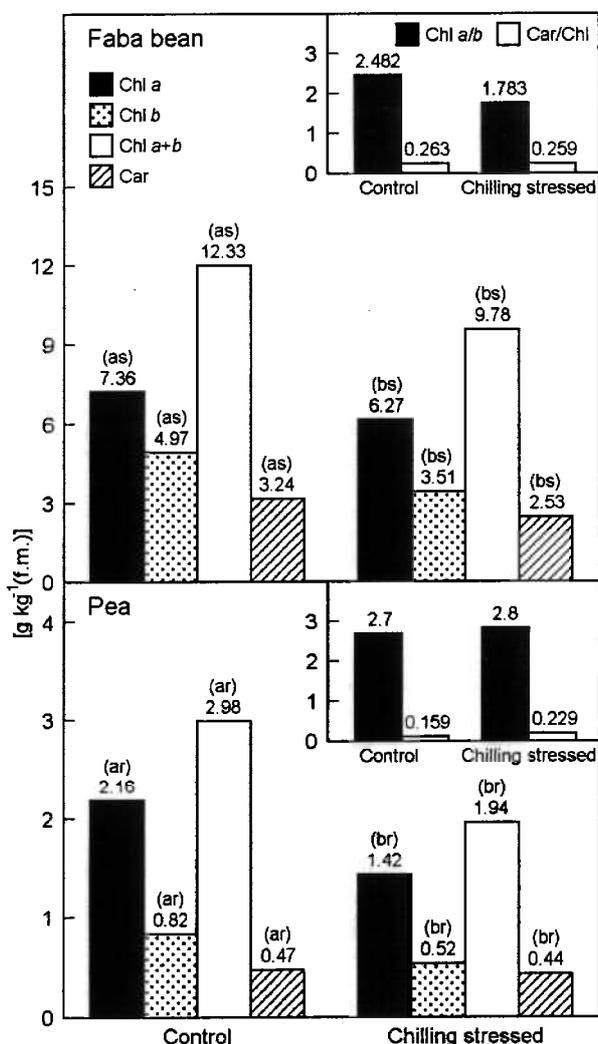


Fig. 1. Concentrations of photosynthetic pigments. A general inhibition of chlorophyll (Chl) and carotenoid (Car) accumulation occurred in chilling stressed faba bean and pea.

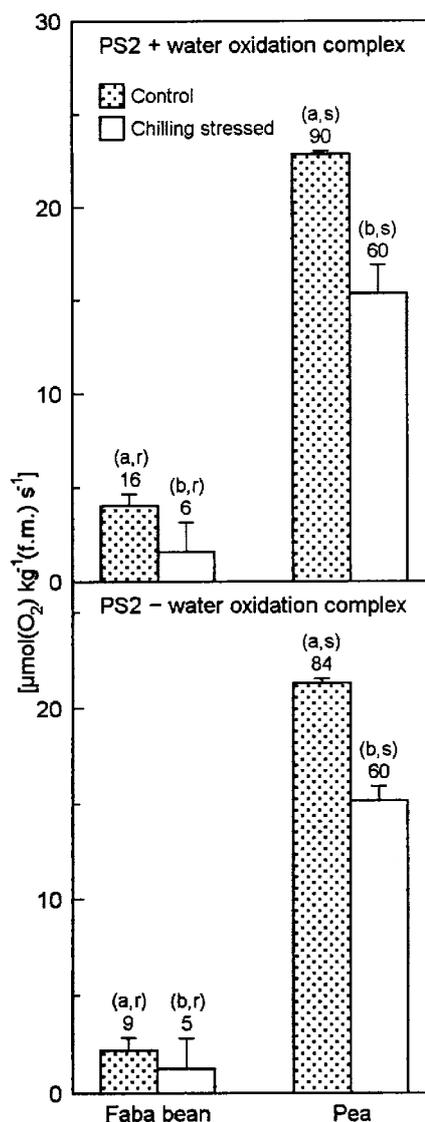


Fig. 2. Photosynthetic electron transport rates coupled to photosystem 2 (PS2). In chilling stressed faba bean and pea a general inhibition of the electron transport chain was found.

respectively (Fig. 1). In both plant species the contents of Chl *a* and *b* also decreased significantly, however, their ratio declined in faba bean but increased in pea (Fig. 1). The concentration of total Cars displayed similar trends, achieving significant decreases under chilling stress (to 78.0 and 93.4 % in faba bean and pea, respectively) (Fig. 1). Nevertheless, the relative proportions between Cars and Chls did not vary significantly in faba bean, but showed a 1.44-fold increase in pea (Fig. 1). In both species the contents of α + β -carotene and lutein+lutein-5,6-epoxide remained unchanged but the contents of violaxanthin+antheroxanthin were depressed in stressed pea (to 47.4 %), whereas zeaxanthin reached 2.1-fold increase (Table 1). Concerning the de-epoxidation state that involves components of the xanthophyll cycle (DEPS), only in chilling stressed pea a 1.55-fold increase was found (Table 1). The photosynthetic electron transport

associated to PS2 (with and without the water oxidation complex) was reduced in both plant species upon chilling (Fig. 2). These inhibitions were, however, more pronounced in faba bean (decline to 39.5 and 48.7 %, respectively) than in pea (decrease to 66.7 and 71.4 %, respectively). In both chilling stressed species the quinone pool diminished whereas the content of low potential form of cytochrome *b*₅₅₉ increased significantly and the concentration of cytochrome *b*_{559HP} remained unchanged (Table 1). The degradation of chloroplast lamellae, although the peroxidation of acyl lipids was higher in faba bean, did not vary significantly under chilling (Table 1). Moreover, in pea a 1.41-fold increase was found. The concomitant protein/Chl ratio was enhanced in both plant species but this tendency was more evident in faba bean (Table 1).

Table 1. Relative proportions of carotenoids and DEPS [%], photosynthetic electron carriers [mmol mol⁻¹(Chl)], lipid peroxidation [mol(MDA) kg⁻¹(Chl)], and protein/chlorophyll ratio in chilling stressed and non-stressed faba bean and pea plants. Means of triplicates \pm S.E..

Control	Faba bean		Pea	
	Control	Stressed	Control	Stressed
α + β carotene	36 \pm 4 ^{ar}	33 \pm 2 ^{ar}	22 \pm 2 ^{as}	22 \pm 3 ^{as}
Lutein+lutein-5,6-epoxide	20 \pm 4 ^{ar}	24 \pm 3 ^{ar}	54 \pm 6 ^{as}	57 \pm 5 ^{as}
Violaxanthin+antheroxanthin	30 \pm 1 ^{ar}	29 \pm 1 ^{br}	19 \pm 1 ^{as}	9 \pm 1 ^{bs}
Zeaxanthin	14 \pm 1 ^{ar}	14 \pm 1 ^{br}	5 \pm 0 ^{as}	11 \pm 0 ^{bs}
DEPS	0.489 ^{ar}	0.486 ^{br}	0.406 ^{ar}	0.628 ^{br}
Quinone pool	26 \pm 2 ^{ar}	15 \pm 2 ^{br}	32 \pm 2 ^{ar}	18 \pm 3 ^{br}
Cyt <i>b</i> _{559LP}	1.11 \pm 0.12 ^{br}	1.92 \pm 0.20 ^{ar}	1.41 \pm 0.12 ^{br}	2.40 \pm 0.21 ^{ar}
Cyt <i>b</i> _{559HP}	1.74 \pm 0.23 ^{ar}	1.65 \pm 4 ^{ar}	2.21 \pm 0.19 ^{ar}	2.37 \pm 0.21 ^{ar}
Lipid peroxidation	41.6 \pm 2.0 ^{ar}	43.0 \pm 2.2 ^{ar}	1.82 \pm 0.13 ^{as}	2.57 \pm 0.14 ^{bs}
Protein/chlorophyll ratio	6.2 \pm 0.7 ^{ar}	13.6 \pm 1.4 ^{ar}	28.2 \pm 1.60 ^{bs}	24.9 \pm 1.4 ^{as}

Discussion

The utilisation of excitation energy in carbon metabolism was reduced when faba bean and pea were transferred from optimal growth temperatures to chilling stress conditions. This process therefore triggered over-excitation of photosynthetic apparatus leading to photoinhibition. As seen by the concurrent decrease of Car concentration, in both species the modelling action of chilling temperatures in conjunction with excess photon amount was expressed by a significant depression of the interacting Car excited state properties. Destructive reactions of singlet oxygen *via* direct quenching of singlet oxygen formation were therefore enhanced (Cogdell and Frank 1987, Lidon and Henriques 1993b). The concomitant effect was a general decrease found for the concentration of photosynthetic pigments due to the irreversible bleaching of Chls, implicating the formation of singlet oxygen (Telfer *et al.* 1991). The structural change that occurs on conversion of violaxanthin to

zeaxanthin in the oxidation of epoxide groups that form cyclohexenyl ring increases the number of conjugated carbon-carbon double bounds, switching the Car from being an antenna of excitation to Chls to its being an acceptor (and quencher) of Chl singlet energy (Owens *et al.* 1992). This influences the apparent polarity of the molecule as well as its ability to establish pigment-pigment interactions. In this context, the high de-epoxidation state involving the components of xanthophyll cycle of pea chloroplasts minimised the yield of photogenerated reactive molecules, mainly in the light-harvesting Chl-protein 2 (Demmig-Adams *et al.* 1989, Lidon and Henriques 1993b). Moreover, the impairment found in the xanthophyll cycle of faba bean minimised the suppression mechanisms of photoproducted reactive molecules, allowing additional production of photon triplet excited Chl and di-oxygen. The unchanged concentrations of carotene and lutein+lutein-5,6-epoxide

followed a tendency previously reported (Thayer and Björkman 1990, Ramalho *et al.* 1997), eventually also supporting the findings of Siefertmann-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. In both chilling stressed species the impairment of photosynthetic electron transport associated to PS2 showed a similar feature indicating that the energy dependent quenching was strongly affected. The general failure of photosynthetic light reactions (even without the water oxidising complex) was much larger in faba bean, which expressed the unchanged rate of the de-epoxidation state that involves the components of xanthophyll cycle. In both chilling stressed species, the strong inhibition of these reactions also paralleled with a decreased accumulation of the quinone pool, further suggesting that the acceptor side of PS2 was coupled to involve high singlet oxygen synthesis; this implicates the formation of the P680 triplet state. This detrimental reaction was probably favoured through the radical pair recombination following the double reduction of quinone A (Vass *et al.* 1992) and its subsequent release from the binding site (Vass *et*

al. 1988, Styring *et al.* 1990). This assumption has additional support considering that the increasing contents of the low potential form of the cytochrome *b*₅₅₉ also point to a photodamaging target at the acceptor side of PS2. Indeed, under these circumstances the related electronic potential allows its oxidation, thus being poised to act as an electron acceptor from reduced phaeophytin. Moreover, the unchanged contents of the high potential form of cytochrome *b*₅₅₉ indicate that photodamage triggered by cation radicals in the reaction centre is not a dominant process (Canaani and Havaux 1990, Rees and Horton 1990, Buser *et al.* 1992). This affects the donation pathway from water to P680 (Erixon and Butler 1971, Lidon and Henriques 1993b) or implicates degradation of the oxygen-evolving complex (Buser *et al.* 1990, Lidon and Henriques 1993b). As seen through the malondialdehyde-thiobarbituric acid complex photodamage, triggered by chilling temperatures affects in a higher extent the structure of pea thylakoid probably due to the interaction of photogenerated oxy-radicals with unsaturated acyl lipids, promoting the synthesis of lipid peroxides. Nevertheless, the increasing ratio between protein and Chl indicates a higher proteolysis in faba bean.

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