**ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ**

**ΣΧΟΛΗ ΘΕΤΙΚΩΝ ΚΑΙ ΤΕΧΝΟΛΟΓΙΚΩΝ ΕΠΙΣΤΗΜΩΝ**
**ΤΜΗΜΑ ΒΙΟΛΟΓΙΑΣ**

**ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ**
**«ΜΟΡΙΑΚΗ ΒΙΟΛΟΓΙΑ ΚΑΙ ΒΙΟΤΕΧΝΟΛΟΓΙΑ ΦΥΤΩΝ»**

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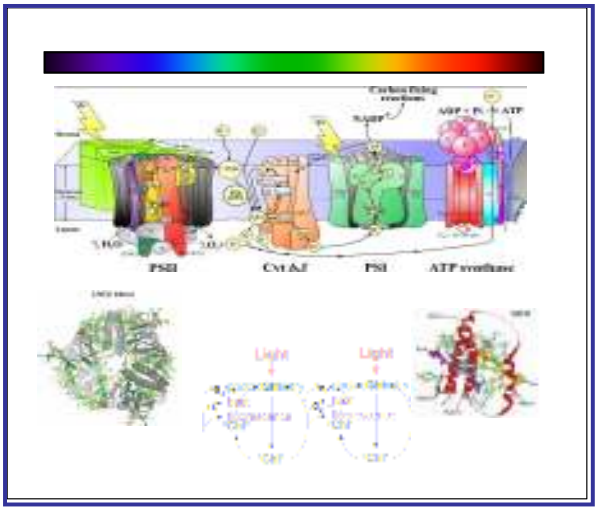
# ΡΥΘΜΙΣΤΙΚΟΙ ΜΗΧΑΝΙΣΜΟΙ ΤΗΣ ΕΥΑΙΣΘΗΣΙΑΣ/ΑΝΘΕΚΤΙΚΟΤΗΤΑΣ ΤΟΥ ΦΩΤΟΣΥΝΘΕΤΙΚΟΥ ΜΗΧΑΝΙΣΜΟΥ ΣΤΗΝ UVB ΑΚΤΙΝΟΒΟΛΙΑ

# REGULATORY MECHANISMS OF THE PHOTOSYNTHETIC APPARATUS SENSITIVITY/TOLERANCE TO UVB RADIATION

*LILIANA V. SFICHI*

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Από

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Η ΠΑΙΔΕΙΑ ΣΤΗΝ ΚΟΡΥΦΗ
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# CONTENTS

# ABBREVIATIONS

## TERMS OF GENETIC SIGNIFICANCE

| | |
|---|---|
| ***wt*** | Wild type strain of *Scenedesmus obliquus* synthesizing both Chl a and b and, therefore, it has LHCII |
| ***wt-lhc*** | Mutant strain of *Scenedesmus obliquus* synthesizing only Chl a and, therefore, it has no LHCII |

## TERMS OF PHYSICAL SIGNIFICANCE

| | |
|---|---|
| **UVB** | Ultraviolet B radiation : 280-320 nm |
| **PAR** | Photosynthetically active radiation : 400-700 nm |
| **ML** | Monochromatic visible light |
| **D** | Darkness |
| **LL** | Low light intensities of PAR (<100 μmol $m^{-2}$ $s^{-1}$) |
| **HL** | High light intensities of PAR (>600 μmol $m^{-2}$ $s^{-1}$) |

## TERMS OF BIOLOGICAL SIGNIFICANCE

| | |
|---|---|
| **PSII** | Photosystem II |
| **PSII-α** | Photosystem II with big LHCII, localised in the grana thylakoids |
| **PSII-β** | Photosystem II with small LHCII, localised in the stroma thylakoids |
| **PSI** | Photosystem I |
| **Cyt $b_6$/f** | Cytochrome $b_6$/f |
| **RC** | Reaction center |
| **LHCII** | Major light-harvesting complex of PSII |
| **CP** | Chlorophyll-protein complex |
| **OEC** | Oxygen evolving complex |

## TERMS OF BIOCHEMICAL SIGNIFICANCE

| | |
|---|---|
| **Chl a** | Chlorophyll a |
| **Chl b** | Chlorophyll b |
| **Chl a/b** | The ratio between Chl a amount and Chl b amount, which is indicative of the LHCII size |
| **PChlide** | Protochlorophyllide |
| **Car** | Carotenoid |
| **α-car** | α-carotene |
| **β-car** | β-carotene |
| **Nx** | Neoxanthin |
| **L** | Lutein |
| **Lx** | Loroxanthin |
| **Vx** | Violaxanthin |
| **Zx** | Zeaxanthin |
| **Ax** | Antheraxanthin |
| **Vx+Ax+Zx** | β-xanthophyll pool |
| **Vx/(Ax+Zx)** | Expression describing the de-epoxidation of Vx into Ax and Zx |
| **L+Lx** | α-xanthophyll pool |
| **Lx/L** | Expression describing the de-epoxidation of Lx into L |
| **$P_{680}$** | primary electron donor of PSII (reaction center of PSII) |
| **$Q_A$** | Primary quinone acceptor of PSII |
| **$Q_B$** | Second quinone acceptor of PSII |
| **PQ** | Plastoquinone pool |
| **Put** | Putrescine |
| **Spd** | Spermidine |

| | |
|---|---|
| **Spm** | Spermine |
| **AOS** | Active oxygen species |

## TERMS OF BIOPHYSICAL SIGNIFICANCE

| | |
|---|---|
| **Fo** | Initial fluorescence (measured when the reaction centers are open, at O step of the fluorescence induction curve) |
| **Fm** | Maximal fluorescence (measured when the reaction centers are closed, at P step) |
| **O, J, I, P** | Steps of the Chl a fluorescence rise |
| **Fo'** | Initial fluorescence of light-adapted sample, measured after far red illumination of a sample previously irradiated with continuous red light and saturated pulses given at determined time intervals |
| **Fm'** | Maximal fluorescence of light-adapted sample, measured at steady-state of Chl fluorescence, acquired after illumination of a sample with continuous red light and saturated pulses given at determined time intervals |
| **Fs** | Steady-state of Chl fluorescence of light-adapted sample, measured at steady-state of Chl fluorescence, acquired after illumination of a sample with continuous red light and saturated pulses given at determined time intervals |
| **Fv/Fm** | Maximum quantum yield of PSII photochemistry, estimated after dark-adaptation from Fo and Fm |
| **$\Phi_{SPSII}$** | Operational quantum yield of PSII photochemistry, calculated in light adapted state from Fm' and Fs |
| **ETR** | The rate of electron transport measured in light-adapted state |
| **qP** | Photochemical quenching capacity |
| **1-qP** | Expression describing the excitation pressure exerted on PSII |
| **qN** | Non- photochemical quenching capacity |
| **qEmax** | Capacity for high-energy non- photochemical quenching |
| **qT** | Non-photochemical quenching due to state transitions |
| **qI** | Non-photochemical quenching by photoinactivated reaction centers |
| **qPQ** | Fluorescence parameter expressing the fluorescence quenching due to oxidized PQ |
| **ABS/RC** | Absorbance per active reaction center - a measure for the functional antenna size |
| **DIo/RC** | Rate of energy dissipation per active reaction center |
| **TRo/RC** | Efficiency of exciton trapping per reaction center |
| **RC/CS** | Density of active reaction centers per cross section |

| | |
|---|---|
| **PSIo** | Efficiency with which an exciton can move an electron further than $Q_A^-$ in the electron transport chain |
| **PI** | Performance index of PSII |
| **pG** | Overall grouping probability - a measure to estimate the energetic connectivity between photosynthetic units |

**TERMS OF PRACTICAL ASSAY SIGNIFICANCE**

| | |
|---|---|
| **DCFH-DA** | Dichlorofluorescein diacetate |
| **DCMU** | 3-(3,4-dichlorophenyl)-1,1-dimethylurea |

**TERMS OF TECHNICAL SIGNIFICANCE**

| | |
|---|---|
| **HPLC** | High-performance liquid chromatography |
| **TLC** | Thin-layer chromatography |
| **PAM fluorometry** | Pulse-amplitude modulated fluorometry |
| **PEA** | Plant Efficiency Analyzer |
| **PCV** | Packed cell volume |

# SUMMARY

The depletion of stratospheric ozone over the Antarctic and Arctic has been observed since 1974 and 1990, respectively. The principal consequence of stratospheric ozone depletion is the increase in ultraviolet B (UVB: 280-320 nm) radiation reaching the Earth's surface. The potential impact of the enhanced solar UVB radiation predicted by atmospheric models has been the subject of investigation for the last two decades. The data collected from various reports involves roughly 300 species and varieties of plants. Around one-third to one-half of these plants showed physiological damage and/or growth reductions in response to UVB. Many studies have identified PSII as the most labile component of the photosynthetic apparatus to elevated UVB radiation. Still now the underlying mechanisms are a controversial subject that makes difficult to evaluate the environmental relevance of UVB effects on photosynthesis. Several different target sites have been proposed. These include the reaction center of PSII, the light harvesting complex (LHCII) and the acceptor/donor side of PSII. In spite of the great amount of research devoted to the effects of UVB radiation on plants during the past decades, efforts are still needed to clarify the molecular background of the UVB damage, as well as the protective and repair mechanisms. The primary target of UVB radiation in the photosynthetic apparatus is not clearly established. In addition, there are discrepancies between laboratory and field studies that make it difficult to estimate how much the projected increase in UVB radiation at the Earth's surface will affect photosynthesis. In this context, this work was focused on the factors determining the sensitivity/tolerance of the photosynthetic apparatus to UVB and their regulation. The investigations were carried out in cultures of *wt* and *wt-lhc* mutant (similar to *wt* but without LHCII) of *Scenedesmus obliquus*. The results demonstrate that there is a fine mechanism that regulates the photosynthetic behavior to UVB radiation. This mechanism adjusts the molecular structure, conformation and function of the photosynthetic apparatus to UVB through regulation of the LHCII antenna. When exposed to UVB irradiation, which increases the over-excitation of PSII reaction centers, the photosynthetic apparatus adopts a behavior that simulates photoadaptation to low PAR

(photosynthetically active radiation: 400-700 nm) intensities. Moreover, this UVB-induced alteration is strongly affected by the PAR background used during UVB treatment. Low PAR (LL) intensities increase the susceptibility of the photosynthetic apparatus to UVB damage, whilst high PAR (HL) intensities confer certain degree of protection, making the photosynthetic apparatus more tolerant to UVB stress. Furthermore, the synergistic action of LL or the antagonistic action of HL with UVB radiation is related to the changes in the thylakoidal Put/Spm ratio that adjust the oligomerization status of LHCII. The overall conclusion is that polyamine changes in the thylakoid membranes act as a primary mechanism which adjusts the degree of sensitivity of the photosynthetic apparatus to UVB radiation by regulating the size of the functional antenna and therefore the photochemical and non-photochemical quenching of absorbed energy. UVB simulates the same molecular and bioenergetic changes that characterize the adaptive response of the photosynthetic apparatus to low light intensities. This means a low Put/Spm ratio in thylakoids which leads to increase in the LHCII size, inactivation of reaction centers and, therefore, enhanced non-photochemical quenching. Photoadaptation to high light conditions induces exactly the opposite changes (high Put/Spm ratio in thylakoids leads to a LHCII size decrease, activation of reaction centers and, subsequently, to increased photochemistry rates). Therefore, HL adaptation acts antagonistically to the UVB effect and enhances the tolerance against UVB radiation. In contrast, LL adaptation amplifies the UVB effect and minimizes the tolerance and enhances the sensitivity to UVB. In fact, PAR intensity influences the excitation pressure of PSII, which adjusts the balance of Put and Spm levels in thylakoids and especially in LHCII forms. Comparative experiments with *wt* and *wt-lhc* mutant (similar to *wt* but without LHCII) cultures confirmed that the sensitivity/tolerance of a photosynthetic organism depends on the LHCII characteristics. Series of action spectra and the difference of action spectra between *wt* and *wt-lhc* cultures (Δ(*wt*–*wt-lhc*)) showed clearly that three primary photoreceptors (active and inactive PChlide (620-640/442 nm), an unknown carotenoid absorbing at 535 nm and the reaction center of PSI (690-730 nm) increase the tolerance of the photosynthetic apparatus to UVB by inducing responses that simulate HL adaptation and subsequently reduce the over-excitation exerted on PSII by UVB. To the contrary, chlorophylls (Chl a and b) are the primary photoreceptors

responsible for the enhanced sensitivity of the photosynthetic apparatus against UVB radiation by increasing the excitation pressure exerted on PSII. Another important finding of the present study is that a photosynthetic apparatus without LHCII has no potential to recover the UVB-induced damage. Moreover, even when there is LHCII, recovery is strictly expressed under light conditions. Cultures incubated in darkness during UVB treatment have no potential to restore the PSII activity affected by UVB. The practical importance of the present study consists in the fact that through artificial changes of the Put/Spm ratio (exogenous supplied polyamines) it is possible to simulate LL-adapted or HL-adapted photosynthetic apparatus and therefore organisms are absolutely tolerant or sensitive towards UVB, independent from the ambient light conditions. In the context of projected increases in the levels of UVB radiation reaching the Earth's surface, as predicted by different scenarios of ozone depletion, data presented herein conclusively indicate that the photosynthetic apparatus possesses the tools and mechanisms to adapt to stress by adjusting the polyamine pattern and subsequently the balance between energy absorption and dissipation. Good examples of this conclusion are the plants grown in the Mediterranean region, generally or, more specifically, in Crete. Although in Crete plants receive daily a great amount of solar energy (high PAR and UVB intensities), their growth is not affected by UVB because the intense photosynthetic capacity in a high PAR environment counteracts the harmful effects of UVB radiation.

# ΠΕΡΙΛΗΨΗ

Η μείωση του στρατοσφαιρικού όζοντος πάνω από τις Ανταρκτικές και Αρκτικές περιοχές διαπιστώθηκε για πρώτη φόρα το 1974 και το 1990, αντίστοιχα. Το σημαντικότερο αποτέλεσμα αυτής της αλλαγής είναι η αύξηση της υπεριώδους ακτινοβολίας (UVB: 280-320nm) που προσπίπτει στην επιφάνεια της Γης. Η πιθανή επίδραση της αυξημένης UVB ακτινοβολίας στην βιόσφαιρα και ιδιαίτερα στα φυτά αποτέλεσε αντικείμενο εντατικής έρευνας τις τελευταίες 2 δεκαετίες. Τα αποτελέσματα μέχρι στιγμής αφορούν συνολικά 300 είδη φυτών. Περίπου στο ένα τρίτο έως και στα μισά από αυτά παρουσιάστηκαν έντονα συμπτώματα καταπόνησης, που σε μερικές περιπτώσεις οδήγισαν ακόμη και σε μείωση της αύξησης τους. Πολλές μελέτες ανέδειξαν το φωτοσύστημα II (PSII) ως το πιο ευαίσθητο σημείο του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία. Ως στόχοι της UVB ακτινοβολίας στο PSII έχουν προταθεί κατά καιρούς το κέντρο αντίδρασης του PSII, το σύμπλοκο της φωτοσυλλεκτικής κεραίας (light-harvesting complex, LHCII) καθώς επίσης και οι ηλεκτρονιοδέκτες/ηλεκτρονιοπομποί του PSII. Παρ'όλα αυτά μέχρι σήμερα δεν γνωρίζουμε τον πρωτογενή μηχανισμό, ο οποίος ρυθμίζει την απόκριση του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία. Αυτός είναι και ο κύριος λόγος που δεν μπορούν να εκτιμηθούν οι επιπτώσεις της προβλεπόμενης αύξησης της UVB ακτινοβολίας στην επιφάνεια της Γης, καθώς και οι ασυμφωνίες μεταξύ των αποτελεσμάτων από διάφορες μελέτες, που έγιναν έως σήμερα. Η παρούσα ερευνητική εργασία επικεντρώθηκε στην μελέτη των διαφοροποιήσεων της μοριακής δομής και λειτουργίας του φωτοσυνθετικού μηχανισμού, οι οποίες επηρεάζουν την ευαισθησία/ανθεκτικότητα του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία, καθώς επίσης και στους μηχανισμούς ρύθμισης τους, αποσκοπώντας στην ανάδειξη του πρωτογενή μηχανισμού απόκρισης στην UVB ακτινοβολία. Οι πειραματικές προσεγγίσεις έγιναν με καλλιέργειες αγρίου τύπου (*wt*) και με το μετάλλαγμα *wt-lhc* (φωτοσυνθετικός μηχανισμός παρόμοιος του *wt*, αλλά χωρίς LHCII) του μονοκύτταρου χλωροφύκους *Scenedesmus obliquus*. Τα αποτελέσματα έδειξαν ότι υπάρχει ένας εξαίρετος μηχανισμός, ο οποίος ρυθμίζει την μοριακή δομή, διαμόρφωση και λειτουργικότητα του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία, μέσω του LHCII. Όταν ο φωτοσυνθετικός μηχανισμός

εκτίθεται στην UVB ακτινοβολία, η οποία αυξάνει την «πίεση» διέγερσης του PSII, ο φωτοσυνθετικός μηχανισμός εμφανίζει αλλαγές, οι οποίες θυμίζουν φωτοπροσαρμογή σε χαμηλές εντάσεις φωτισμού. Αυτή η συμπεριφορά φάνηκε να επηρεάζεται έντονα από την ένταση της ορατής ακτινοβολίας (PAR-ακτινοβολία) στην διάρκεια της έκθεσης του οργανισμού σε UVB καταπόνηση. Συγκεκριμένα, οι χαμηλές εντάσεις φωτισμού αυξάνουν σημαντικά την ευαισθησία του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία, ενώ οι υψηλές εντάσεις φωτισμού την μειώνουν, αυξάνοντας σημαντικά την ανθεκτικοτήτα του. Βιοχημικές μελέτες έδειξαν ότι ο λόγος για τον οποίο οι χαμηλές εντάσεις φωτισμού δρουν συνεργιστικά, ενώ οι υψηλές εντάσεις φωτισμού δρουν ανταγωνιστικά με την UVB ακτινοβολία, οφείλεται πρωτίστως σε αλλαγές του επιπέδου των συνδεδεμένων στα θυλακοειδή πολυαμινών και ιδιαίτερα του λόγου Put/Spm, οι οποίες ακολουθούνται από αλλαγές στη σχέση ολιγομερών/μονομερών του LHCII. Η διαφοροποίηση των πολυαμινών στα θυλακοειδή φαίνεται να αποτελεί την πρωτογενή απόκριση του φωτοσυνθετικού μηχανισμού στην UVB, ρυθμίζοντας τον βαθμό ευαισθησίας του φωτοσυνθετικού μηχανισμού δια μέσου ρύθμισης του μεγέθους και της σύστασης του LHCII. Ρυθμίζοντας το μέγεθος της λειτουργικής φωτοσυλλεκτικής κεραίας, οι πολυαμίνες ρυθμίζουν τη χρήση της συλλεχθείσας ηλιακής ενέργειας στις φωτοχημικές και μη-φωτοχημικές διαδικασίες του φωτοσυνθετικού μηχανισμού. Η UVB ακτινοβολία προσομοιώνει τη μοριακή δομή και λειτουργία ενός φωτοσυνθετικού μηχανισμού προσαρμοσμένου σε χαμηλής έντασης ακτινοβολία (μείωση του λόγου Put/Spm, αύξηση του μεγέθους της φωτοσυλλεκτικής κεραίας, μείωση των ενεργών κέντρων αντίδρασης του PSII, αύξηση ως εκ τούτου της μη-φωτοχημικής απόσβεσης της ενέργειας, κλπ.). Αυτός είναι και ο λόγος που οι χαμηλές εντάσεις φωτισμού (PAR-ακτινοβολίας) δρουν συνεργιστικά με την UVB ακτινοβολία εντείνοντας την επιδρασή της. Σε αντίθεση με τις χαμηλές εντάσεις, η φωτοπροσαρμογή του φωτοσυνθετικού μηχανισμού σε υψηλές εντάσεις φωτισμού δρα ανταγωνιστικά της UVB, λόγω του ότι σηματοδοτεί αλλαγές στη μοριακή δομή και λειτουργία του φωτοσυνθετικού μηχανισμού, ακριβώς αντίθετες από αυτές της UVB ακτινοβολίας (αύξηση του λόγου Put/Spm, μείωση του μεγέθους της φωτοσυλλεκτικής κεραίας, αύξηση των ενεργών κέντρων αντίδρασης του PSII, μείωση ως εκ τούτου της μη-φωτοχημικής και αύξηση της φωτοχημικής απόσβεσης της ενέργειας, κλπ.), αυξάνοντας σημαντικά την ανθεκτικότητα του φωτοσυνθετικού

μηχανισμού και ως εκ τούτου και του οργανισμού, στην UVB ακτινοβολία. Εξωγενής προσθήκη πουτρεσίνης (αύξηση της σχέσης Put/Spm) σε καλλιέργειες προσαρμοσμένες σε συνθήκες χαμηλού φωτισμού μείωσε την επίδραση της UVB και η ανθεκτικότητα του φωτοσυνθετικού μηχανισμού στην UVB αυξήθηκε. Αντίστοιχα, προσθήκη εξωγενούς σπερμίνης (Spm) σε συνθήκες υψηλού φωτισμού όπου η επίδραση της UVB δεν ήταν τόσο έντονη όπως στις συνθήκες χαμηλού φωτισμού, προκάλεσε αύξηση στην ευαισθησία του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία. Συγκριτικές μελέτες του *wt* με το μετάλλαγμα *wt-lhc* έδειξαν ότι η ευαισθησία του φωτοσυνθετικού μηχανισμού στην UVB εξαρτάται από τις δομικές και βιοενεργητικές διαφοροποιήσεις του LHCII. Σειρές από φάσματα δράσης και στις δύο καλλιέργειες ( *wt* και *wt-lhc*) και ο υπολογισμός της διαφοράς στην απόκρισή τους στην UVB κάτω από διαφορετικές συνθήκες μονοχρωματικής ακτινοβολίας, έδειξαν ότι υπάρχουν τρεις πρωτογενείς φωτοϋποδοχείς [το ενεργό (640/442nm) και ανενεργό (640/442nm) πρωτοχλωροφυλλίδιο (PChlide), ένα άγνωστο καροτενοειδές (535nm), και το ενεργό κέντρο αντίδρασης του φωτοσυστήματος I (690-730nm)], που ρυθμίζουν την αύξηση της ανθεκτικότητας του φωτοσυνθετικού μηχανισμού στην UVB ακτινοβολία, επάγοντας μια διαφοροποίηση της μοριακής δομής και λειτουργίας του φωτοσυνθετικού μηχανισμού ίδια μ'εκείνη που συναντάται σε συνθήκες υψηλής έντασης ακτινοβολίας. Σε αντίθεση, οι χλωροφύλλες (Chl a & b) αναδεικνύονται ως οι πρωτογενείς φωτοϋποδοχείς, που οδηγούν στην αύξηση της ευαισθησίας του φωτοσυνθετικού μηχανισμού στην UVB μέσω αύξησης του μεγέθους του LHCII. Ένα άλλο σημαντικό αποτέλεσμα από την παρούσα μελέτη είναι ότι μόνο παρουσία LHCII και φωτισμού, ο φωτοσυνθετικός μηχανισμός έχει την ικανότητα να επανέλθει στην αρχική του κατάσταση μετά το πέρας της UVB καταπόνησης. Συνοψίζοντας, τα αποτελέσματα που παρουσιάζονται σ'αυτή την εργασία δείχνουν ότι τα φυτά διαθέτουν τα μέσα και τους μηχανισμούς προστασίας τους από τη UVB ακτινοβολία προσαρμόζοντας τα επίπεδα των συνδεδεμένων στα θυλακοειδή πολυαμινών. Αυτό ρυθμίζει στην συνέχεια το μέγεθος και τη διαμόρφωση του LHCII και διαμέσου αυτού την ενέργεια που χρησιμοποιείται στην φωτοχημεία και στη μη-φωτοχημεία σε στενή σχέση με τις περιβαλλοντικές συνθήκες φωτισμού. Όλα τα παραπάνω μας επιτρέπουν να κατανοήσουμε τον λόγο που φυτά που μεγαλώνουν σε περιβάλλοντα με υψηλή ένταση (ορατής) ακτινοβολίας, αλλά και υψηλή ένταση UVB ακτινοβολίας, όπως αυτό της Κρήτης, δεν επηρεάζονται από τις

βλαβερές δράσεις της UVB λογω της ανταγωνιστικής δράσης της υψηλής έντασης της PAR-ακτινοβολίας.

# INTRODUCTION

# INTRODUCTION

## 1. STRATOSPHERIC OZONE DEPLETION AND UVB IMPACT ON GLOBAL CLIMATE

One billion years ago, early aquatic organisms called blue-green algae began using solar energy to convert $H_2O$ and $CO_2$ molecules into organic compounds and molecular oxygen ($O_2$) through a process now called photosynthesis. Some of the photosynthetically created oxygen is combined with organic carbon to produce $CO_2$ molecules. The remaining oxygen accumulated in the atmosphere and touched off a massive ecological disaster with respect to early existing anaerobic organisms. As oxygen in the atmosphere increased, $CO_2$ decreased. In stratosphere, some oxygen molecules absorbed energy from the sun's ultraviolet (UV) rays and split to form single oxygen atoms in a process called photolysis. These atoms combined with the remaining $O_2$ molecules to form ozone ($O_3$) molecules, which are very effective at absorbing UV rays. The thin layer of ozone (compressed it would only be 3 mm, the thickness of two stacked pennies) that surrounds Earth acts as a shield of 20-60 km height and protects the planet from harmful effects of UV light. The amount of ozone required to shield Earth from biologically lethal UV radiation is believed to have an existence of 600 million years. Prior to this period, the oxygen level was approximately 10% of its present atmospheric concentration and life was restricted to the ocean. The presence of stratospheric ozone enabled organisms to develop and live on the land.

Solar radiation is divided into UV (ultra-violet, 8 % of the total energy), visible light (39 %) and infrared radiation (53%) (Coohill, 1989). Traditionally, UV is divided into three wavelength ranges:

- **UVC** (200–280 nm) is extremely harmful to organisms, but not relevant under natural conditions of solar irradiation, since it is absorbed in the upper layers of the atmosphere;
- **UVB** (280–320 nm) is of particular interest because this wavelength represents only approximately 1.5% of the total spectrum, but can induce a variety of damaging effects in plants;
- **UVA** (320–400 nm) represents approximately 6.3% of the incoming solar radiation and is the less hazardous part of UV radiation.

As sunlight passes through the atmosphere, all UVC and approximately 90% of UVB radiations are absorbed by stratospheric ozone, water vapours, oxygen and carbon dioxide. Therefore, the UV radiation reaching the Earth's surface is largely composed of UVA with a small UVB component (Fig.1).

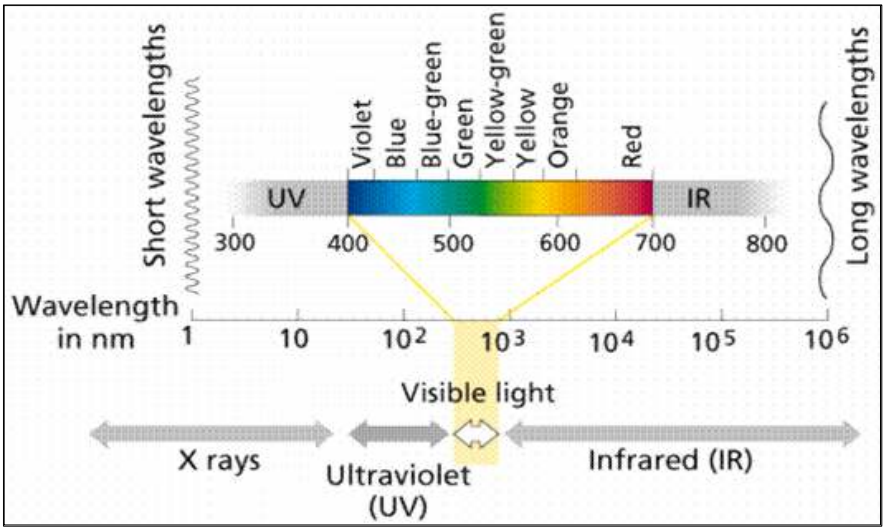


**FIGURE 1.** Solar light spectrum composition (*Source*: NASA)

The concentration of ozone in the upper atmosphere has been declining since the 1970's. The main reason for the ozone depletion is the pollution of stratosphere by CFC (chlorofluorocarbons), compounds containing carbon, chlorine (freons) or bromine (halogens). Because of catalytic reactions, an individual chlorine atom can on average destroy nearly a thousand ozone molecules before it is converted into a form harmless to ozone. When UV strikes CFC* ($CFCl_3$) molecules in the upper atmosphere, a carbon-chlorine bond breaks, producing a chlorine (Cl) atom. The chlorine atom then reacts with an ozone ($O_3$) molecule breaking it apart and so destroying the ozone. This forms an oxygen ($O_2$) and a chlorine monoxide (ClO) molecule. Then a free oxygen atom breaks up the chlorine monoxide. The chlorine is free to repeat the process of destroying more ozone molecules. A single CFC molecule can destroy 100 000 ozone molecules. The property that makes ozone good for filtering UV radiation makes it also easily destroyed: it is very unstable. A NASA spectrometer has detected an Antarctic ozone "hole" (what scientists call an "ozone depletion area") that is three times larger than the entire land mass of the United States. The "hole" expanded to a record size of approximately 30.3 x 106 $km^2$ in

2000. Today, some scientists are predicting that the stratospheric ozone layer will recover to 1980 ozone levels by the year 2050 (Schrope, 2000), but no recovery signs are visible yet.

Ozone's impact on climate consists primarily of changes in temperature. The more ozone in a given parcel of air, the more heat it retains. Ozone generates heat in the stratosphere, both by absorbing the sun's UV and by absorbing infrared radiation from the lower atmosphere (troposphere). Consequently, decreased ozone layer in the stratosphere results in lower temperatures, which in turn contribute to $O_3$ destruction. As the ozone layer gets thinner, UV radiation at the surface of the Earth increases. If the ozone amount decreases by 10% during the spring and summer, the annual UV dose increases by about 12%. Increases in UVB have been already observed in Antarctica during the ozone hole development as well as in the northern hemisphere (Tevini, 2004).

On physicochemical basis, the energy contribution of UVB is of minor importance. However, on the basis of its photobiological effects, it is highly important to the Earth's biosphere. Since plants in a natural environment are unavoidably exposed for long time periods to UVB, it is often a source of considerable stress to them (Caldwell, 1971; Jordan, 1996). The potential impact of these predicted UVB increases on plant physiology has been subjected to investigation for the last two decades, and the data collected from various reports roughly involve 300 species and varieties of plants. Nearly one-third to one-half of these plants showed physiological damage and/or growth reductions in response to UVB (Teramura and Sullivan, 1994).

## 2. MOLECULAR TARGETS OF UVB RADIATION

UVB effects have been studied during the last thirty years in small growth cabinets, growth chambers, and greenhouses or in the field supplementing white light or ambient solar UV radiation with artificial UVB or attenuating (or even excluding) the UVB from the solar light. Supplementation studies were useful elucidating UV stress responses and accompanying mechanisms; however, they are less reliable in providing estimates for natural habitants, where many environmental parameters such as water and mineral stress and/or high sun light irradiances are often interfering. Attenuation of solar UVB by appropriate filters, such as ozone or plastic films, avoids the use of artificial UV (Tevini and Teramura, 1989; Mark et al., 1996). However, by

this method only relative UVB enhancements compared to the reduced solar UVB can be evaluated. The studies conducted until presently revealed that UVB acts on macromolecules and molecules of biological importance, perturbing the processes in which they are involved as described below.

## 2.1. NUCLEIC ACIDS

DNA is one of the most notable targets of UV. Irradiation in both the UVB and UVC regions results in a multitude of DNA photoproducts (Sancar and Sancar, 1988), which may cause mutations during replication (Jiang and Taylor, 1993). The most common DNA photoproducts are cyclobutane-type pyrimidine dimers (CPDs) and the pyrimidine(6,4)pyrimidone dimer (6,4 photoproduct) (Hutchinson, 1987). DNA protein cross-links, and DNA strand breaks and deletion or insertion of base pairs can also be induced by UV exposure (Smith, 1989). UV-induced damages to DNA have been studied in detail in humans, mammals, fungi and bacteria (Stapleton, 1992).

## 2.2. AMINO ACIDS AND PROTEINS

Proteins have strong absorption at about 280 nm, as well as, at higher wavelengths of the UVB region due to absorption by the aromatic amino acids phenylalanine, tryptophane and tyrosine, as well as histidine, cysteine and cystine and, therefore, can be direct targets of UV-radiation. UV-induced destruction of tyrosine and tryptophane has been observed both in the form of free amino acids and proteins. UVB can induce photooxidation of tyrosine and the formation of di-tyrosine. Photochemical changes initiated in tryptophane are due to its excitation either directly by UV or by energy transferred from neighbouring amino acids such as tyrosine or phenylalanine. Cysteine is a relative poor absorber in the UVB region, but undergoes UV-induced photolysis at high quantum efficiency. The disulfide group of cystine can be split by UV radiation into reactive sulfhydryl groups influencing protein tertiary structure and function (Creed, 1984). UV radiation may induce the inactivation of whole proteins and enzymes by photolysis of aromatic amino acids or disulfide groups, if the affected residues are included in the active site. It is also important to note that UV absorption within the protein matrix may cause damage via energy migration to functionally important amino acids of an active center, as suggested for

the sensitization of cysteine destruction by aromatic residues (Jordan, 1993), as is the case of ribulose 1,5-bisphosphate carboxylase (Rubisco) (Vu et al., 1984), ATP-ase, violaxanthin de-epoxidase (Pfündel et al., 1992) and protein subunits of the photosystem I and II ( Jordan, 1993). Protein components of the plant cytoskeleton may be possible targets for UV radiation. Tubulin may be a particularly sensitive target, since it has a high content of amino acids with aromatic side chains (Zaremba et al., 1984).

## 2.3. LIPIDS

Lipids with isolated or conjugated double bonds can also be photochemically modified by UVB. Phospho- and glycolipids, which are the main components of plant cell membranes, may be affected by the oxidative stress induced by UVB (Panagopoulos et al., 1990). Hydroxyl radicals and singlet oxygen can react with the methylene groups forming conjugated dienes, lipid peroxy radicals and hydroperoxides. Either radicals or singlet oxygen can be produced by photosensitization with dyes, aromatic carbohydrates or porphyrins (Smirnoff, 1995). The peroxy radicals can abstract hydrogen from other polyunsaturated fatty acids, leading to a chain reaction of peroxidation. One of the products of lipid peroxidation is malondialdehyde which is often used as a measure of peroxidation. Hydrogen peroxide can also inactivate enzymes, particularly some of the light-activated Calvin-cycle enzymes (Charles and Halliwell, 1980). Hydroxyl radicals can denature proteins and react with bases in DNA causing mutations. The aldehydes formed by lipid peroxidation can conjugate and inactivate proteins (Wolff et al., 1986). The composition of membrane lipids in chloroplasts such as mono- and digalactosyldiglicerides (MGDG, DGDG) may change due to UVB. Since a high degree of unsaturation of MGDG is necessary for stability of chloroplast membrane structure (Hugly et al., 1989), the decreases in the MGDG/DGDG ratio with UVB may influence membrane stability.

## 2.4. PLANT GROWTH REGULATORS

UV-induced changes in DNA and/or plant growth regulators are the possible reasons for changes in growth, general development and flowering. Photolytic degradation of indole-3-acetic acid (IAA) has been demonstrated in sunflower

seedlings (Ros and Tevini, 1995). The reduced IAA concentration and the growth inhibitor IAA photoproduct, 3-methylene-oxindole, may be responsible for the inhibition of hypocotyl growth. Furthermore, the action of peroxidases, functioning as IAA-oxidases, may also inhibit the elongation since the cell wall extensibility is reduced. Abcisic acid (ABA) also strongly absorbs in the UVB region and can be inactivated by photolysis (Lindo et al., 1979). Growth can be stimulated by gibberellins in UV-irradiated seedlings (Ballaré et al., 1991), while ethylene is produced to a greater extent in UVB irradiated plants (Ros and Tevini, 1995).

### 2.5. PIGMENTS

UVB radiation may induce photobleaching and photodegradation of photosynthetic pigments (Strid and Porra, 1992). Chlorophylls (Chl) and carotenoids (Car) may be adversely affected by relative large amounts of UVB, with carotenoids generally being less affected than chlorophylls (Pfündel et al., 1992). High levels of UVB radiation in combination with low levels of PAR have significantly reduced the chlorophyll content in bean, barley and corn, pea and soybean (reviewed in Teramura and Ziska, 1996). However, the effect of UVB on photosynthetic pigments and Chl a/b ratios varies among growth and irradiation conditions and species (Teramura and Sullivan, 1994; Day and Vogelmann, 1995; Correia et al., 1999).

### 2.6. MEMBRANES

UVB-induced damage to membranes is mainly focused on transport phenomena (Murphy and Vu, 1996). UVB seems to exert adverse effects not only on various protein and pigment-protein complexes of the photosynthetic apparatus, but also on the structure of the thylakoid membrane that contains these complexes. An early consequence of UVB is an increase in ion-permeability of the thylakoid or plasmatic membrane (Chow et al., 1992).

## 3. UVB EFFECTS ON PHOTOSYNTHESIS

Studies of more than 300 plant species and cultivars have been carried out and about 50% have been considered sensitive, 20–30% moderate sensitive and the rest insensitive to UVB radiation (Teramura and Sullivan, 1994). In many sensitive plant species (e.g. wheat, rice, maize, rye, sunflower, and cucumber), reduced leaf area

and/or stem growth was found. Photosynthesis is one of the most studied processes under UVB accompanied mainly by growth experiments.

Despite the diversity of UVB targets in plants, it seems that the photosynthetic apparatus is among the main action sites of UVB and its damage significantly contributes to the overall UVB damage (Kulandaivelu et al., 1993) (Fig. 2). Proteins, photosynthetic pigments, quinones such as plastoquinones and unsaturated fatty acids of galactolipids, all present in the bilayered structure of the thylakoid membrane, may be UVB targets due to their electronic absorption in the UVB region (Tevini, 2004).

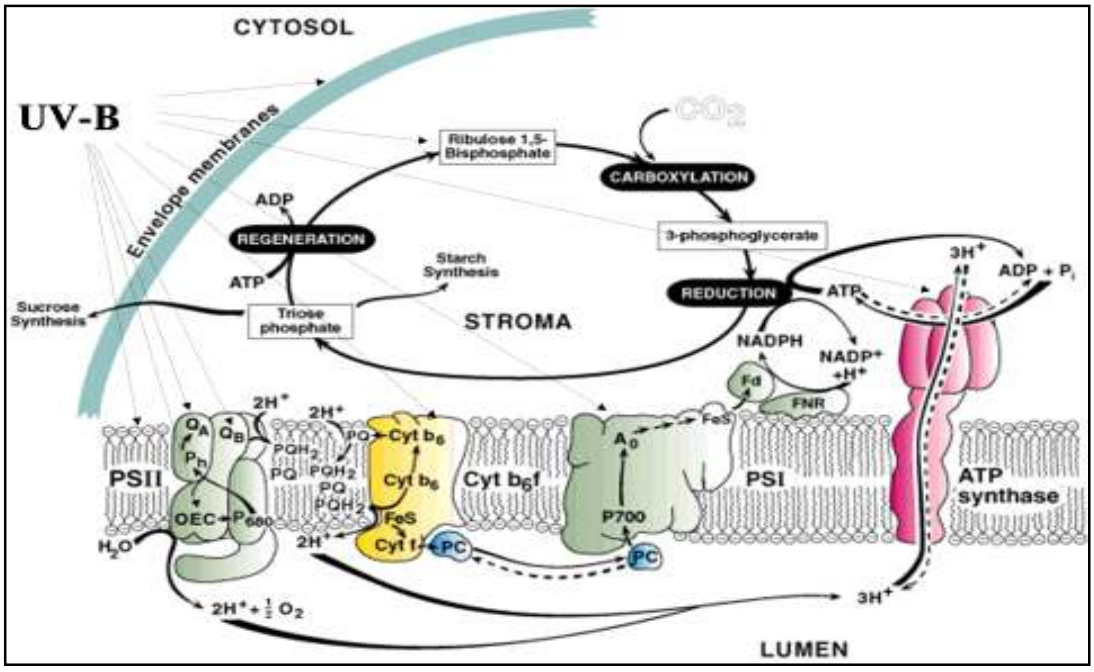


**FIGURE 2.** Molecular targets of UVB radiation in the photosynthetic apparatus

However, it is important to distinguish between direct damage, e.g. by absorption of high energy UV-radiation causing destruction of the molecules itself and indirect damage by reactive oxygen species (ROS) produced during the destruction. For example, ROS oxidize polyunsaturated fatty acids and generate reactive fatty acid peroxides, which further react with synthetic pigments. Furthermore, it was found in green leaves that ROS may down-regulate the expression of photosynthetic genes (Mackerness et al., 1999). Thus, it is not clear whether reductions in photosynthetic pigments as often found under high UVB irradiances (Strid and Porra, 1992; Kim et al., 1996) are due to direct destruction or to

biosynthetic defects following DNA damage. Direct damage to unsaturated membrane lipids was concluded from the formation of malondialdehyde (Kramer et al., 1991). An action spectrum for this lipid peroxidation process shows a shift more to the UVC region and less effectiveness in the UVB one (Cen and Björn, 1994).

The direct effects of enhanced UVB radiation in sensitive plants (as reviewed in Jansen et al., 1998) are the following:

• impairment of photosystem II (PSII) and to lesser extent photosystem I (PSI);

• decreased Rubisco activity;

• decreased carbon dioxide fixation and oxygen evolution;

• reduction in dry weight, starch and chlorophyll content.

In addition to the direct effect of UVB radiation, photosynthesis may also be indirectly affected by:

• induction of stomatal closure, which may reduce the efficiency of gas exchange;

• changes in leaf thickness and anatomy, which may alter light environment within the leaf;

•changes in canopy morphology, which may also indirectly affect whole plant photosynthesis.

## 3.1. MOLECULAR TARGETS OF UVB RADIATION IN THE PHOTOSYNTHETIC APPARATUS

Within each chloroplast, the photosynthetic thylakoids membranes form a physically continuous three-dimensional network that encloses a single aqueous space, the thylakoid lumen. The most striking feature of mature thylakoid membranes is their differentiation into grana and stroma regions, also called stacked or appressed and unstacked or non-appressed regions, respectively (Fig. 3) (Mustárdy and Garab, 2003). Three-dimensional models of the spatial relationship between grana and stroma thylakoids show that PSII and LHCII reside mainly in the grana membanes, while PSI and ATPase reside predominantly in the stroma membanes and the cytochrome $b_6$/f complex is distributed about evenly between the two types of membranes (Dekker and Boekema, 2005). All the four complexes (PSII, PSI, cyt$b_6$/f and ATP-ase), as well as, Rubisco and thylakoid membranes have been found to be targets of UVB radiation (Fig. 2).

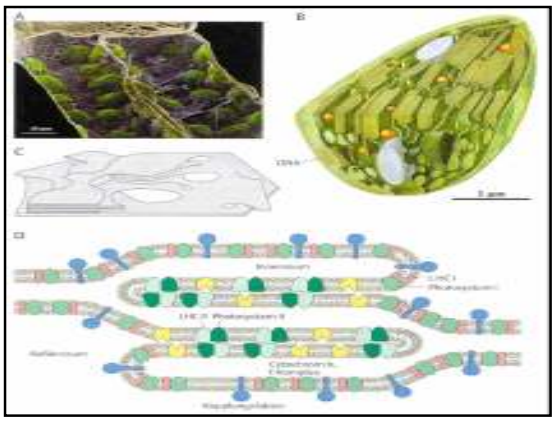

**FIGURE 3.** Thin-section electron micrograph of a higher plant chloroplast and the schematic representation of the spatial distribution of photosynthetic apparatus components on thylakoid membranes (*Source:* Mustardy and Garab, 2003)

### 3.1.1. UVB impact on photosystem II (PSII)

PSII is the membrane protein complex found in oxygenic photosynthetic organisms (higher plants, green algae and cyanobacteria) (Fig. 4), which harnesses light energy to split $H_2O$ into $O_2$, protons and electrons (Anderson and Styring, 1991). It drives one of the most oxidizing reactions known to occur in nature and is responsible for the production of atmospheric oxygen, essential for aerobic life on this planet. In addition, by catalysing the first step of the photosynthetic electron transport chain, PSII is also involved in the production of a substantial portion of the global biomass. In higher plants and green algae well over 20 subunits are associated with PSII *in vivo* and have been named after the genes encoding them (*PsbA-PsbY*, *Lhcb1-Lhcb6*). The location and organization of the genes that encode these proteins has been reviewed in detail (Erickson and Rochaix, 1992).

The photochemically active reaction center (RC) consists of the D1 and D2 proteins with molecular masses of 38 kD and 39.4 kD. The cofactors associated with the D1 and D2 proteins include a 4 atom Mn cluster, $P_{680}$, pheophytin (Pheo), $Q_A$, $Q_B$, non-heme iron, accessory Chls and β-carotene (Barber et al., 1987; Nanba and Satoh, 1987; Tang et al., 1990). Closely associated with the reaction center are the two largest PSII subunits, CP47 (*PsbB*) and CP43 (*PsbC*), which form an antenna within the core complex. Their biochemical characterization was recently reviewed (Bricker and Ghanotakis, 1996). One of the functions of CP43 is thought to be the transfer of excitation energy from the Chl a/b binding proteins to the PSII reaction center (Bassi and Dainese, 1992), whereas mutagenesis studies suggest that CP43 may be partially involved in providing an environment for the Mn cluster (Kuhn and Vermaas, 1993).

In higher plants and green algae the oxygen evolving complex (OEC), which is closely associated with the Mn cluster of PSII (Gilchrist et al., 1995) is formed by 3 subunits (33 kD, 23 kD and 16 kD).

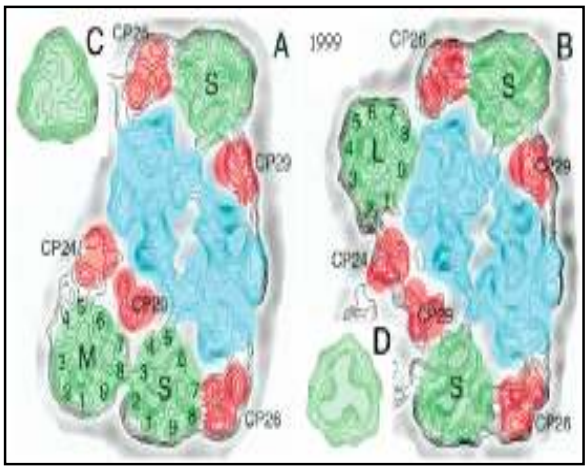


**FIGURE 4.** The subunits of PSII and their organization in different types of super-complexes (*blue*: reaction center with the core antenna; *green*: LHCII trimers) (*Source*: Boekema et al., 1999).

The Z-scheme describes the path of electron transfer from water to NADP+ and depicts each carrier at its midpoint redox potential on a vertical scale with the more reducing (negative Em) components on the top and the more oxidizing (positive Em) on the bottom (Fig. 5). The Mn cluster catalyses water oxidation and electrons liberated during this process are transferred to the reaction center chlorophyll, $P_{680}$, via a redox active tyrosine residue, Tyr-Z, of the D1 protein. PSII contains another redox-active tyrosine, called Tyr-D, on the D2 subunit, which can donate electrons to $P_{680}$, but not connected to the water-oxidizing complex. On the acceptor side of PSII, the electron produced by the light induced charge separation event, reduces a pheophytin molecule and then the first ($Q_A$) and second ($Q_B$) PQ electron acceptors (Andersson and Styring, 1991). $Q_A$ is a firmly bound component of the reaction center complex, which undergoes one electron reduction, whereas $Q_B$ is a mobile electron carrier, which takes up two electrons sequentially from $Q_A$ before leaving its binding site formed by the D1 protein (Fig.5). While there is general consensus that UVB radiation influences primarily PSII, there are many different reports on possible targets (Tevini, 2004). Different techniques were used to reveal the possible target sites of UVB radiation such as fluorescence induction, flash-induced absorption changes, and measurement of oxygen evolution.

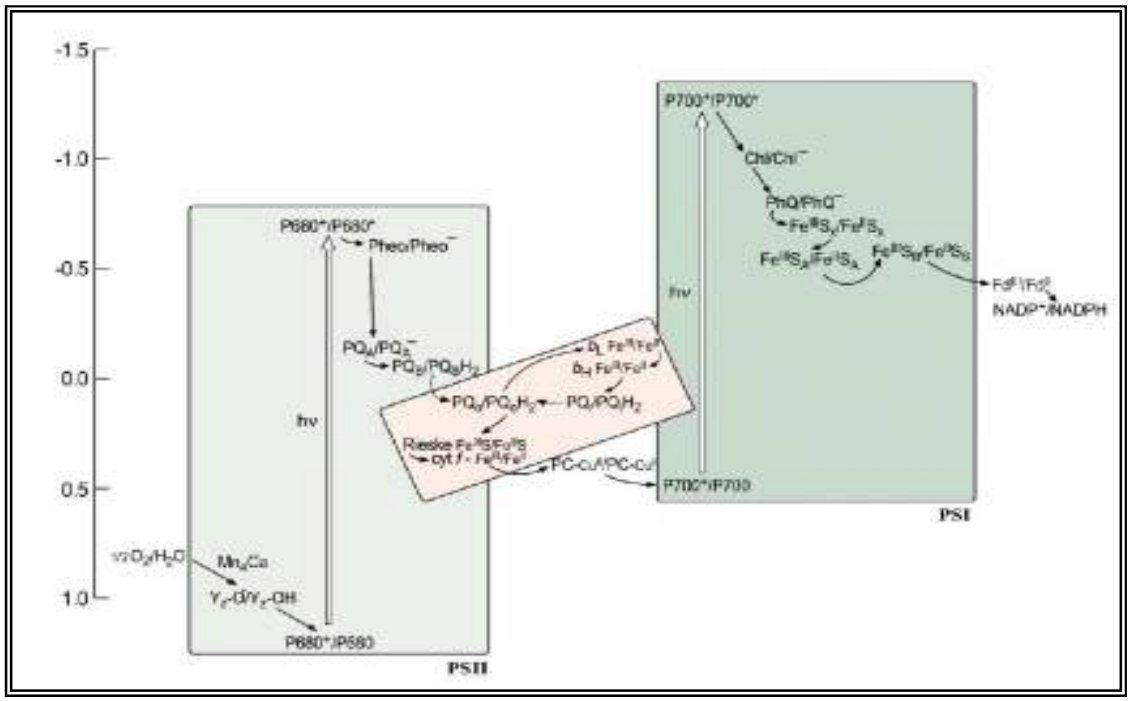


**FIGURE 5.** An up-dated Z-scheme describing photosynthetic electron transfer from $H_2O$ to NADPH (*Source*: Merchant and Sawaya, 2005).

Critical comparison of the published data is often complicated by the essentially different experimental conditions: spectral composition of the applied UV source (presence of UVC component besides UVB or UVA radiation), the presence or absence of visible light and different (visible) light intensities and qualities. However, it seems to be well established that the redox components of PSII are affected by UVB to some degree (Fig. 2). From previous experiments it has been assumed that UVB acts on either the reaction center itself, producing dissipative sinks for excitation energy, which quenches the variable fluorescence and/or the reducing site of PSII (Iwanzik et al., 1983). Recent comparative studies indicated the water-oxidizing complex as the most UV-sensitive part of PSII (Bornman and Sundby-Emanuelsson, 1995). Since the Mn cluster of water oxidation seems to be the most fragile component of the electron transport chain, UVB absorption by the protein matrix or other redox components may lead to conformational change and inactivation of the Mn cluster. Most observations support the notion that UVB preferentially inactivates the water-oxidizing complex with additional effects on the $Q_A$ and $Q_B$ acceptors, as well as on the Tyr-Z and Tyr-D donors (Renger et al., 1989; Vass et al., 1996; Giacometti et al., 1996). The acceptor or reducing side of the D1 and D2 proteins can

be modified by UVB radiation with a subsequent change in the number and activity of quinone binding sites (Renger et al., 1989). Specifically, it has been suggested that UVB radiation primarily modifies the binding sides on the PSII acceptor side with a simultaneous blocking of pheophytin, the primary electron acceptor (Renger et al., 1986). UVB radiation also decreases chlorophyll (Chl) flurorescence with the fast components accelerated and the slow components retarded, suggesting the formation of additional quenchers of exciton energy in reaction centers (Renger et al., 1991). It has been indicated that plastoquinone with its three redox states (quinone, semiquinone anion and the quinol) may act as a primary UVB photosensitive molecule since all these forms absorb to the same extent in the UVB region (Melis et al., 1992). Recently, it was shown that UVB induces both structural and excitonic uncoupling of Chl within the light-harvesting complexes. Transient absorption measurements and low-frequency infrared and Raman spectroscopy show that the predominant sites of UVB damage in PSII are at the OEC itself, as well as at specific locations near the OEC-binding sites (Lukins et al., 2005).

A combination of high intensity PAR and UVB radiation results in enhanced rates of photodamage and degradation of the D1 protein (Greenberg et al., 1989), although UVB driven protein cleavage occurs at different sites as compared to that induced by PAR and is thought to be independent of the presence of oxygen (Melis et al., 1992; Barbato et al., 1995). Under supplemental UVB, both D1 and D2 proteins are subject to photodamage (Melis et al., 1992; Jansen et al., 1993; Friso et al., 1994; Vass et al., 1996). The interplay between PAR and supplemental UVB radiation and the role of the latter in photodamage and turnover of the D1 and D2 proteins are questions of current interest (Masi and Melis, 1997).

### 3.1.2. UVB impact on light harvesting complex II of PSII (LHCII)

PSII is surrounded by its light-harvesting antenna which is comprised of the inner minor antenna complex (built by CP24, CP26 and CP29, encoded by the genes *lhcb4*, *5* and *6*) and the outer major antenna complex LHCII. The structure and function of LHCII has been extensively studied. Its role is not restricted to the capture of photons but also has been attributed to protection of the photosynthetic apparatus against excessive energy flow by its capability to dissipate the excess energy by a

mechanism called non-photochemical quenching, which is activated in a timescale of minutes (Dau, 1994; Horton et al., 1999; Ort, 2001; Li et al., 2004; Pascal et al., 2005). LHCII function is a topical area of research, not only because of its prevalence and light-harvesting role, but also because it is a key target of several signal transduction pathways that control light energy use. It is composed of three polypeptides, termed Lhcb1, 2 and 3, which form homo- or hetero-trimers capable of higher order of polymerization of seven to eight trimers around each PSII (Kühlbrandt et al., 1994; Boekema et al., 1999; Jackowski et al., 2003).

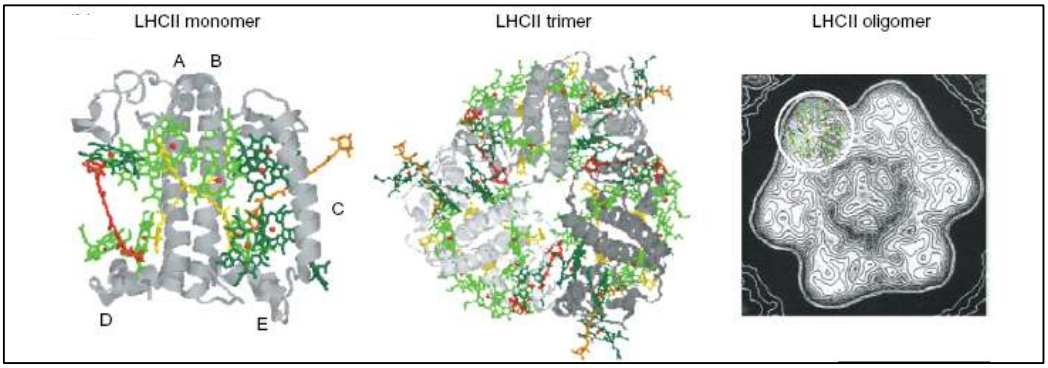


**FIGURE 6.** Aggregation states of LHCII: the monomeric state (*left*, in the membrane plane), the trimeric state (*middle*, seen from above the membrane) and the organization of LHCII trimers in oligomeric forms (*right*; one trimer is indicated by the white circle) (*Source*: Robert et al., 2004).

LHCII exists also in a monomeric form (Fig. 6). It has been established that trimers are subjected to proteolytic degradation yielding monomers (Anastassiou and Argyroudi-Akoyunoglou, 1995; Yang et al., 1995; Navakoudis et al., 2006). Since LHCII plays an important role in light absorption and energy transfer to the reaction center as well as thylakoid stacking, any damage to these system results in multiple effects on the photosynthetic apparatus. UVB radiation decreases the transcription of the *cab* genes responsible for the synthesis of the chlorophyll a/b-binding proteins of LHCII and may lead to the functional disconnection of LHCII from PSII (Jordan et al., 1994). In addition, it was showed that the increase in *pr1* transcript and decrease in *Lhcb* transcript in response to UVB exposure in *Arabidopsis thaliana* is mediated

through pathways involving hydrogen peroxide derived from superoxide anion (Mackerness et al., 2001).

### 3.1.3. UVB impact on photosystem I (PSI)

PSI structure was revealed a few years ago in cyanobacteria (Jordan et al., 2001). The structure of the monomer showed 12 Psa subunits (named as gene products *PsaA*, *PsaB*, *PsaC*, etc.) and 127 cofactors arranged in symmetrical pairs bound by a heterodimeric core made up of the homologous polypeptides PsaA and PsaB. Ferredoxin associates with PSI on the stromal side via interaction with PsaC, PsaD, and PsaE. Plastocyanin and cytochrome c6 associate with PSI on the lumen side via interaction with PsaA, PsaB, and PsaF. Most of the pigment molecules in PSI, 90 chlorophyll a molecules and 22 carotenoids, function in the core antenna system. The PSI reaction is initiated by excitation of the primary donor, $P_{700}$, via energy transfer from the core antenna and the electron is sequentially transferred to ferredoxin (Merchant and Sawaya, 2005) (Fig. 5).

The effects of UVB radiation do not seem to be evenly distributed between the two photosystems. Based on a variety of experiments, a general consensus has emerged that UVB has little or no effect on PSI (Kulandaivelu and Noorudeen, 1983) as compared to PSII (Renger et al., 1989; Bornman, 1989). UVB radiation may inhibit PSI-mediated cyclic photophosphorylation (Pang and Hays, 1991). Recently, it was found that UVB induces a decrease in the PSI efficiency. This effect may result from enhanced charge recombination in the reaction center, which might represent an incipient inactivation of PSI, but contributes to thermal dissipation of excessive light energy and thereby to photoprotection. (Krause et al, 2003). However, the possible targets within PSI have not been studied in detail.

### 3.1.4. UVB impact on cytochrome $b_6$/f (cyt $b_6$/f) complex

Cyt $b_6$/f mediates electron transport between the two photosystems: it oxidizes plastoquinol produced by PSII and reduces plastocyanin, which serves as electron donor to PSI. The enzyme occurs as a functional dimmer. There are four large subunits, cytochrome $b_6$, cytochrome f, the Rieske protein, and subunit IV that bind the redox cofactors and four smaller ones, PetG, PetL, PetM, and PetN. The position of electron transfer cofactors in the cyt $b_6$f complex is compatible with the Q-cycle

mechanism, which was devised to account for the ratio of protons pumped per quinone oxidized (discussed in Allen, 2004; Smith et al., 2004). The substrate plastoquinol is bound at the Qo site, where its oxidation and proton release into the lumen takes place. One electron from the quinol is transferred to the Rieske ironsulfur center from where it is transferred to the c-heme of cytochrome f and then eventually to plastocyanin or cytochrome $b_6$. The other electron from the quinol at the Qo site is transferred via the two b-hemes to a stromal side quinone at the Qi site where proton uptake from the stroma and reduction takes place (Fig. 5). Cyt $b_6$/f complex together with PSI seems to be the least affected part of the thylakoid membrane by UVB radiation (Cen and Bornman, 1990).

### 3.1.5. UVB impact on the Rubisco and the ATP-ase

Supplemental UVB radiation may decrease the activity and content of the PSII complex with a resulting decrease in electron transport and presumably ATP synthesis (Teramura and Ziska, 1996). Rubisco activity may decline with enhanced levels of UVB radiation (Vu et al., 1984; Strid et al., 1990). High UVB irradiance in combination with low PAR levels produces significant reduction in the concentration of carboxylating enzymes (Barbato et al., 1995). When high PAR (>1000 $\mu mol^{-2}\ s^{-1}$) was applied in conjunction with low UVB levels the Rubisco was unaffected. UVB induced reduction of photosynthetic capacity was related to a reduction in the initial carboxylation velocity of Rubisco which was further correlated with a large reduction in the expression and abundance of both large and small subunits of Rubisco (Jordan, 1993; Keiller et al., 2003). UVB-induced inactivation of Rubisco could be due to modification of the peptide chain, degradation of the protein and/or diminished transcription of the gene (Jordan et al., 1994). Recently, *in vivo* photomodification of Rubisco holoenzyme was observed in UVB-treated plants (Wilson et al., 1995). There is also a marked decline in the amount and activity of ATP-synthase in UVB irradiated plants (Zhang et al., 1994).

## 3.2. GENERAL RESPONSES TO UVB RADIATION IN PLANT PHOTOSYNTHESIS

Although the effect of UVB on carbon reduction is not directly mediated by the diffusion of $CO_2$ through the leaf, supplemental UVB can indirectly limit the

photosynthetic capacity by inducing stomatal closure (Wright and Murphy, 1981). However the response of stomata to UVB radiation may be dependent upon prevailing environmental conditions. Stomata close upon direct exposure to UVB, but if strong white light is used, stomata can re-open immediately (Negash and Björn, 1986). UVB radiation in combination with low PAR appears to have the greatest impact on stomatal closure (Mirecki and Teramura, 1984). UVB radiation can also reduce photosynthetic activity by reducing leaf area with subsequent decrease in light interception. Leaf area appears to be particularly sensitive to increased UVB radiation, especially in a background of low PAR levels (Teramura and Ziska, 1996). Recently, it was shown that *Brassica napus* grown under above ambient levels of supplemental UVB radiation exhibited an increase in overall leaf width, although no change in leaf anatomy was discerned. Thylakoid stacks were broader and shorter in leaves subjected to UVB. In general these responses were similar to those which occurred in plants moved from a high to low PAR environment (Fagerberg and Bornman, 2005).

In the last decade several reviews of UVB effects on plant photosynthesis have been published (Teramura and Sullivan, 1994; Fiscus and Booker, 1995; Teramura and Ziska, 1996; Tevini, 2004). Primary productivity and photosynthesis have been measured in several terrestrial ecosystems such as forests, dune grassland or tropical plants (Tevini, 2004). Negligible effects on maximum quantum yield of PSII (Fv/Fm) have been observed under enhanced UVB added to solar UVB. In Mediterranean pines, UVB seems to be even beneficial to enhanced UVB at least in periods of water stress where the cuticle thickness increases and the stomata close. Other species did not show any negative effects of UVB on photosynthesis (Nogues and Baker, 2000). These results demonstrate that many of the negative or damaging effects elaborated under artificial UV radiation may be ameliorated under field conditions, where in addition to ambient or artificially enhanced UVB the background white light repairs damage or increases content of protective pigments (Searles et al., 2001). On the other hand, attenuation studies clearly show that higher UVB can impact on plant morphology and phenology when compared to lower ambient UVB (Tevini, 2004). Whether UVB radiation is a stress factor depends on wavelength, irradiance and exposure time, as well as, on the genetic, morphological and protective predisposition of the plant species or cultivar (Krizek, 2004). Analysis of leaf proteome after UVB irradiation in maize lines differing in sensitivity to UVB demonstrated that the

differential regulation of proteins by UVB could be genetically fixed traits conferring UVB tolerance and adaptations to living in high ambient UVB conditions (Casati et al., 2005).

## 4. PROTECTION AGAINST UVB RADIATION

### 4.1. PROTECTIVE STRUCTURES

In order for UVB radiation to be effective in altering plant biochemistry, physiology or productivity, it must penetrate the plant to sensitive targets and be absorbed by chromophores. Thus, epidermis represents an important protective barrier for plants against harmful effects of UV radiation (Tevini et al., 1991), since it is often ornamented with hairs and trichomes and may contain UV-absorbing compounds (Skaltsa et al., 1994). Other surface characteristics, such as dense glaucescence or epicuticular wax may enhance epidermal reflectance and in some cases UV radiation can act on the wax composition itself (Tevini and Steinmüller, 1987). For most plant species, the reflectance of the surfaces in the UV range has secondary importance (less than 5%) and most of the attenuation can be attributed to scattering and absorption of light (Robberecht and Caldwell, 1978). However, there are few species in which the leaf surface reflectance lies within a range of 20–70% in the UV region (Robberecht et al., 1980). Experiments with optical fibers also revealed that there are differences between different leaf surfaces (abaxial and adaxial) concerning the penetration of either monochromatic (310 nm) or polychromatic UVB (280-320 nm) radiation (Cen and Bornman, 1990). Furthermore, increased leaf thickness, often observed in UV-irradiated plants, may result from increased length of individual cells attenuating visible and UVB light.

### 4.2. PROTECTIVE AND REPAIR MECHANISMS

The overall UVB sensitivity of the cells is determined by the balance between the damage that occurs and the efficiency of the repair processes that can restore the impaired functions.

#### 4.2.1. Repair of DNA damage

Plants have evolved three different repair mechanisms to minimize the UVB-induced injury to the genetic material. UV radiation-induced DNA damage can be

repaired by photoreactivation, excision, or recombinational repair (Britt, 1995; Taylor, 1996). Pyrimidine cyclobutane dimers (CPDs) induced by UVB can be repaired by all of these mechanisms. During photoreactivation repair a light-requiring repair enzyme, photolyase, is responsible for the direct splitting of pyrimidine cyclobutane dimers. This enzyme utilizes light energy in the range of 370–450 nm (blue/UVA), to monomerize CPDs. Therefore, the UVA part of the spectrum has an important role in photoreactivation repair. However, an action spectrum for DNA damage in *Medicago sativa* seedlings showed that CPD formation occurs even at wavelengths as long as 365 nm (Quaite et al., 1992). Experimental evidence suggest that *Arabidopsis* may have a light dependent pathway for the repair of pyrimidine(6,4)pyrimidone photoproducts (Chen et al., 1994). Thus, *Arabidopsis* has the ability to photoreactivate both of the major UV-induced DNA damage products. Damage to DNA, other than CPDs, can be repaired by excision or recombinational repair (Sancar and Sancar, 1988; Sutherland et al., 1996). The process of excision repair can be divided into three steps: nicking of the damaged DNA near the site of the damage, removal of bases in the damaged strand and resynthesis of the gap. An endonuclease that is responsible for the nicking of the damaged DNA has been purified from carrot cells (McLennan and Eastwood, 1986). Photoreactivation and excision repair in the dark have been reported in higher plants (e.g. *Daucus*, *Nicotiana*, *Petunia*, *Haplopappus*), but mainly following UVC exposure (McLennan and Eastwood, 1986). Recently, it was demonstrated that homologous recombination repair pathways might be involved in eliminating UVB-induced DNA lesions in plants of *Arabidopsis* and tobacco (Ries et al, 2000).

### 4.2.2. Scavenging of ROS

ROS may play a role in mediating UVB damage (Strid et al., 1994; Rao et al., 1996; Mackerness et al., 1999). In turn, low fluences of UVB induce scavenging capacity by up-regulation of genes encoding enzymatic or non-enzymatic scavengers (Jansen et al., 1996; Rao et al., 1996). The origin of these ROS is unclear but it has been proposed that UVB exposure may lead to ROS generation by increasing NADPH oxidase activity (Rao et al., 1996). Illumination of isolated thylakoids with UVB radiation indicated that UVB does not result in singlet oxygen production but induces free radicals, mainly hydroxyl (Hideg and Vass, 1996). In *Ulva fasciata*, \

alleviation of UVB-induced oxidative damage by a $H_2O_2$ scavenger, dimethylthiourea, and a free radical scavenger, sodium benzoate, suggests that oxidative damage caused by UVB is ascribed to accumulated $H_2O_2$ (Shiu and Lee, 2005). It is proposed that ROS mediate a series of signal transduction pathways each controlling the expression of different specific genes, i.e. up-regulation of pathogenesis-related genes and down-regulation of photosynthetic genes (Surplus et al., 1998). Consequently, it has been concluded that the antioxidant capacity of a plant tissue dictates the relative sensitivity of photosynthetic genes to UVB induced down-regulation (Green and Fluhr, 1995; Surplus et al., 1998; Mackerness et al., 1999). Recently, it was found that a methyl viologen-resistant mutant of *Arabidopsis* is tolerant to supplemental UVB radiation due to enhanced activities of ROS-scavenging enzymes (plastidic Cu/Zn superoxide dismutase and stromal ascorbate peroxidase) in chloroplasts and that the acquired tolerance to the short-term UVB exposure results from a higher accumulation of sunscreen pigments (Fujibe et al., 2004).

### 4.2.3. UVB absorbing compounds

Apart from anatomical alterations of plants, the accumulation of UVB absorbing compounds often found under enhanced UVB in species of natural ecosystems, as well as UVB reflecting waxes, may contribute to the protection of photosynthesis in nature (Tevini and Steinmüller, 1987; Tevini, 2004). Low fluences of UVB stimulate the general phenylpropanoid pathway, resulting in accumulation of flavonoid and sinapic esters (Li et al., 1993; Day and Vogelmann, 1995; Van de Staaij et al., 2002). These compounds play a protective role by specific absorbtion in the wavelength region from 280 to 340 nm (but not in the PAR waveband, which would diminish photosynthetic yields). In barley the accumulation in epidermal and subepidermal mesophyll tissue of flavonoids (saponarin, lutonarin) reduces the UVB-induced DNA damage (Schmitz-Hoerner and Weissenbock, 2003). Flavonoids also possess free radical scavenging activity, which might offer additional protection to cells accumulating these compounds (Rice-Evans et al., 1997). This response could be regarded as a plant strategy to minimize the flux of harmful radiation into crucial parts of plant tissue, such as the photosynthetic apparatus in the chloroplast. Besides flavonoids, the accumulation of carotenoids has protective function against UVB stress (Middleton and Teramura, 1993). In *Dunaliella*, the accumulation of β-carotene

prevents UVB-induced photosynthetic damage through absorption of UVB and BL (White and Jahnke, 2002). Additionally, in transformants of *Synechococcus* PCC79421, the accumulation of β-carotene and zeaxanthin resulted in the reduction of UVB damage, through inactivation of UVB-induced radicals in the photosynthetic membranes (Götz et al., 1999).

### 4.2.4. Protective and repair mechanisms of the photosynthetic apparatus

4.2.4.1. Repair of the PSII damage

Many studies of UVB radiation effects on photosynthesis demonstrated that the photosynthetic activity is slowly and incompletely restored. Restoration of UV-induced disfunction of enzymes is expected to involve *de novo* protein synthesis and/or repair of DNA damage both in the chloroplast and nuclear DNA. Recently, it was found that UVB induced damage to D1 and D2 activity of PSII can be partially restored. D1 protein is rapidly turned over *in vivo* in 30 min (Greenberg et al., 1989; Wilson and Greenberg, 1993). When all the UVB radiation was filtered out from sunlight, the rate of D1 protein degradation was as much as 30% slower than in full sunlight (Greenberg et al., 1989). PAR plays an important role in the recovery of PSII structure and function from UVB stress in plants. It was found that a 20 kDa C-terminal fragment of D1 protein generated during irradiation with UVB light was stable when plants were incubated in the dark, but was degraded when plants were incubated in visible light. In this condition the recovery of photosynthetic activity was also observed (Bergo et al., 2003). Similar results showing that a rapid D1 turnover assures the recovery of the PSII functionality have been obtained for other species of plants (Olsson et al., 2000) and cyanobacteria (Campbell et al., 1998).

4.2.4.2. Dissipation of excess excitation energy

Excess energy impinging on the photosynthetic apparatus is deactivated by photochemical and non-photochemical quenching (NPQ). The latter process is broadly defined as all fluorescence quenching that is not directly related to charge separation. NPQ can be subdivided into three components:

1. qE: energy-dependent quenching. It requires the build-up of a proton gradient and relaxes within seconds to minutes;

2. qI: photoinhibitory quenching. It is caused by photoinhibition and shows very slow relaxation kinetics in the range of hours;
3. qT: quenching due to state transitions when the major light-harvesting complex (LHCII) separates from PSII, thereby reducing the amount of the excitation energy in PSII that can de-excite to fluorescence; it relaxes within tens of minutes (Müller et al., 2001).

Intensive research during the past several years has led to a concept of the role of the ΔpH in qE. A decrease in lumen pH induces qE through protonation of PSII proteins and activation of xanthophyll synthesis via xanthophyll cycle. Together, binding of protons and xanthophylls to specific sites in the PSII antenna causes a conformational change that switches a PSII unit into a quenched state with a short $^{1}$Chl* lifetime and a low fluorescence yield (Gilmore, 1997). There are few studies focused on the dissipation of excess energy by NPQ in UVB irradiated plants. Pftündel et al. (1992) showed that violaxanthin deepoxidase activity in higher plants is inhibited by UVB, a fact that also seems to be true for green algae (Döhler et al., 1997). Contradictory results were found for natural phytoplankton by Döhler and Hagmeier (1997). They reported increased concentrations of diatoxanthin accompanied by decreased amounts of diadinoxanthin induced by a combination of UVA and UVB. The UVB-dependent increase in diatoxanthin was correlated with a concomitant enhancement of non-photochemical quenching of Chl fluorescence and a decrease in the quantum efficiency of oxygen evolution. This indicates that UVB induced diatoxanthin functions in thermal energy dissipation.

4.2.4.3. Polyamines

One of the major damages in plant tissues caused by UVB radiation is the destruction of biomembranes (Tevini et al., 1981; Murphy, 1983). Previous published results showed that polyamines, acting as scavengers of ROS (Bouchereau et al., 1999) may stabilize the membranes by reducing the lipid peroxidation promoted by stressors, such as ozone (Bors et al., 1989) or UVB (Kramer et al., 1991). Additionally, Kramer and co-workers (1992) reported that the intracellular accumulation of polyamines in soybean under UVB radiation is influenced by the intensity and spectral quality of visible light, but data for the UVB effect on polyamines associated to specific organelle, such as chloroplasts, are still missing.

This aspect may become of added concern in the research field of UVB effects on photosynthesis, since polyamines have been found to play an important role in the structure and function of the photosynthetic apparatus.

The three main polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) are compounds with many biological functions such as cell division, growth and senescence (Slockum et al., 1984; Igarashi and Kashiwagi, 2000; Paschalidis and Roubelakis-Angelakis, 2005) due to their cationic nature.

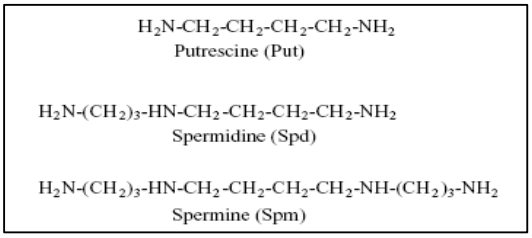


**FIGURE 7.** The three main polyamines: putrescine (Put), spermidine (Spd) and spermine (Spm) found in plants, animals and microbes.

In plants, Put is formed directly by the decarboxylation of ornithine, via ornithine decarboxylase (ODC), or indirectly from arginine by arginine decarboxylase (ADC) via agmatine (Agm) (Tabor and Tabor, 1985; Tiburcio, et al., 1990). Polyamines differ in both the number of positive charges exhibited at the physiological pH of the cell (two in Put, three in Spd and four in Spm) and the backbone length (Spm: 1.46 nm; Spd: 1.112 nm; Put: 0.65 nm) (Fig. 7). The close association of polyamines with macromolecules is generally thought to constitute the physical basis of their numerous modes of action.

During the past decade a number of data have been collected that highlight the involvement of polyamines in photosynthesis (reviewed in Kotzabasis, 1996). It was shown that polyamines are structural compounds of the photosynthetic apparatus, being associated with the LHCII and PSII complex in spinach (Kotzabasis et al. 1993), or conjugated to LHCII proteins, CP24, CP26, CP29 and the large subunit of Rubisco (Del Duca et al., 1994) by transglutaminase (TGase) (Della Mea et al., 2004). Recently, it was reported that TGase activity is light-stimulated in isolated thylakoids and light-enhanced in intact chloroplasts, suggesting a synergism between the two compartments (Dondini et al., 2003). Furthermore, it was demonstrated that polyamines are involved in the assembly and stabilization of photosynthetic

complexes (Dörnemann et al., 1996), being even responsible for the post-translational modification of LHCII apoproteins (Dondini et al., 2003).

A first approach to investigate their mode of action was attempted by experiments in which the endogenous polyamine level was manipulated by exogenous polyamines or polyamine inhibitors (reviewed in Kotzabasis, 1996). Besford et al. (1993) observed that exogenous polyamines supplied to osmotically stressed oat leaves retarded protein degradation, inhibited loss of chlorophyll and stabilized thylakoid membranes. They identified D1, D2, cyt f and Rubisco subunit as proteins that can be stabilized by the addition of exogenous polyamines. Furthermore, it was demonstrated that polyamines are involved in chlorophyll biosynthesis and in the assembly of the photosynthetic apparatus in mutant C-2A' of *Scenedesmus obliquus*, unable to synthesize chlorophylls in darkness. The reduction of Put level by application of 1, 4-diamino-2–butanone resulted in a substantial inhibition of chlorophyll biosynthesis upon illumination. This was accompanied by an increase in the respiration rate and of the photosynthetic activity, suggesting the formation of a photosynthetic apparatus that behaves similarly to one adapted to high PAR (Beigbeder et al., 1995).

An important finding was the fact that the photosynthetic apparatus can be manipulated to adopt a low light or a high light behaviour by artificial changes of the endogenous level of polyamines with inhibitors or exogenously supplied polyamines. Specifically, Kotzabasis et al. (1999) showed that a decrease of the intracellular Put/Spm ratio simulates a low light-photoadapted photosynthetic apparatus, e. g. enlargement of the LHCII accompanied by a decrease of the reaction center density, and a decrease of the maximum photosynthetic rates, respiration and Chl a/b ratios. In contrast, by increasing the Put/Spm ratio, responses that mimic high light-photoadaptation (decrease of the LHCII size, increase in the Chl a/b ratios and photosynthetic rates) can be induced. In addition, the action spectrum of the Put/Spm ratio suggested that the receptor responsible for this response is a blue light photoreceptor.

In recent years, a series of data confirm the regulatory role of the Put/Spm ratio of the photosynthetic apparatus behaviour in different environmental conditions. In this context, it was shown that in conditions of low temperature (Sfakianakis et al., 2006), ozone (Navakoudis et al., 2003) or UVB radiation (Sfichi et al, 2004; Lütz et

al., 2005) the structural and functional adjustments occurring in the photosynthetic apparatus simulate low light-adaptation, when the reduction in the thylakoid-associated Put/Spm ratio is accompanied by an increase in the LHCII size and a subsequent decline in the photosynthetic rate. The importance of these findings is not only of theoretical order, i. e. in the elucidation of mechanisms that are responsible for the photosynthetic apparatus sensitivity to different environmental stresses.

Several additional works have shown that by manipulation of polyamine pattern a more tolerant photosynthetic apparatus to stress conditions can be obtained. For instance, exogenously manipulated increase of the Put levels was efficient in conferring tolerance to UVB (Sfichi et al., 2004) or ozone in an ozone-sensitive cultivar of tobacco (Navakoudis et al., 2003). Furthermore, Logothetis et al. (2003) found that high $CO_2$ concentration treatment is followed by an increase of thylakoid-bound Put that leads to an increase of the active reaction center density combined with a decrease in the LHCII size and the ratio of LHCII oligomers/monomers. This reorganization of the photosynthetic apparatus that simulates high light-adaptation resulted in the enhancement of photosynthetic activity, which in combination with high $CO_2$ concentrations leads to an immense increase of biomass (800 %). Thus, the existence of a common regulatory mechanism of the photosynthetic apparatus behaviour to environment that by its manipulation can improve the performance of the photosynthetic apparatus under various conditions, is an attractive perspective not only for the increase of plant tolerance to stress but also for the improvement of bioproductivity.

# SCOPE OF RESEARCH

In spite of the great amount of research devoted during the past decades to the effects of UVB radiation on plants, efforts are still needed to clarify the molecular background of the UVB damage, the protective and repair mechanisms, as well as, the characterization of the primary photoreceptors that regulate the UVB responses. The primary target of UVB radiation in the photosynthetic apparatus has not been clearly identified. In addition, there is a discrepancy between laboratory and field studies that make it difficult to estimate how much the projected increase of UVB radiation at the Earth's surface will affect photosynthesis.

In the light of the latest findings on the photosynthetic apparatus' response to environmental factors, the present contribution focuses on the elucidation of mechanisms and factors that determinate the sensitivity of the photosynthetic apparatus to UVB. Concretely, the topics studied and the questions posed for an answer through this work are:

1. The study of the changes in the molecular structure, conformation and function of the photosynthetic apparatus upon irradiation with enhanced UVB and their influence by the visible light (PAR) intensity.
2. Is the photoadaptation status of the photosynthetic apparatus the main mechanism that adjusts the tolerance / sensitivity against UVB radiation? If this is the case, a comparative study between *wt* and *wt-lhc* mutant (similar to *wt* but without LHCII) should be able to clarify the role of LHCII in balancing damage and repair in UVB irradiated photosynthetic apparatus.
3. The regulatory role of polyamines on the photoadaptation status of the photosynthetic apparatus (Navakoudis et al., 2006) admonishes the study of polyamine roles in the adjustments of the photosynthetic apparatus sensitivity/tolerance degree to UVB radiation.
4. The characterization of the primary photoreceptor(s) that attenuates and/or amplifies the UVB effects on the photosynthetic apparatus.

All this information will not only provide theoretical data contributing to the understanding of photosynthetic apparatus sensitivity to UVB, but also could provide a practical solution to enhance the photosynthetic apparatus tolerance to UVB radiation stress.

# MATERIAL AND METHODS

# MATERIAL AND METHODS

## 1. ORGANISM AND GROWTH CONDITIONS

The present contribution focused on the investigation of the photosynthetic apparatus sensitivity to UVB radiation in cultures of *Scenedesmus obliquus.* The taxonomical classification is the following:

- Kingdom: *Plantae* Haeckel, 1866 - plants
- Subkingdom: *Viridaeplantae* Cavalier-Smith, 1981 - green plants
- Phylum: *Chlorophyta* auct. - green algae
- Subphylum: *Chlorophytina* Cavalier-Smith, 1998
- Infraphylum: *Tetraphytae* Cavalier-Smith, 1998
- Class: *Chlorophyceae*
- Order: *Chlorococcales*
- Family: *Scenedesmaceae*
- Genus: *Scenedesmus* von Lagerheim, 1882
- Species: *obliquus* (Turbin) Kutzing

*Scenedesmus obliquus* is a common cosmopolitan green alga that often occurs as an almost pure culture in fresh water plankton. Usual habitats are in waters like clean ponds, lakes and rivers.

**FIGURE 1.** Cells of *Scenedesmus obliquus* in the light microscope (*left* and *middle*) and a single cell in the transmission electron microscope (*right*).

Cells commonly occur in colonies as multiples of two, with four or eight cells being most common (Fig. 1). The morphology of the colony can be varied

considerably by varying the medium in which the cells are growing. In a medium with low phosphorus or low salt concentration, *S. obliquus* is induced to grow unicellular, forming around 10 µm long elliptical cells. The cell wall contains mainly cellulose, pectin and the polycarotenoid sporopollenine, which gives it an extremely resistance. The most common mode of reproduction of *S. obliquus* is asexual. The life cycle lasted about 20 h. On the evolutionary scale, it is nearly positioned to *Chlamydomonas* and *Chlorella*. As all *Chlorophyta*, *S. obliquus* has chlorophyll a and b as photosynthetic pigments and forms starch within the chloroplasts. The *Chlorophyta* thus differ from the rest of the eukaryotic algae in forming the storage product in the chloroplast instead of in the cytoplasm. The photosynthetic processes resemble that of higher plants. Since *Scenedesmus obliquus* can be easily cultivated it was an early investigation object for botanists. Thus, in the early 1950$^{th}$ Hans Gaffron discovered the hydrogen metabolism of eukaryotic algae first in *S. obliquus*. Further investigations were encouraged by the generation of dozens of mutants by Norman Bishop and their photo-physiological investigation by Norman Bishop and Horst Senger.

In the present contribution, *wt* strain D3 (Gaffron, 1938) and *wt-lhc* mutant strain (Bishop, 1982) of *S. obliquus* were used. The *wt-lhc* mutant (kindly provided by Prof. Bishop, USA) is an ethylmethanesulphonate-induced phenotype lacking chlorophyll b and the light-harvesting complex, but retaining near-normal photosynthetic capacity. It is adapting to increased light intensity during autotrophic growth by increasing the size of the proximal antennae (Bishop et al., 1989; Senger et al., 1993). Besides the *wt* cultures, the possibility of using of a *wt-lhc* mutant photosynthetic apparatus to assess the behavior of the photosynthetic apparatus to UVB irradiation is a great advantage, giving the opportunity to quantify the contribution of LHCII to the damage and repair processes occurring upon UVB exposure. Both *wt* and *wt-lhc* cultures were heterotrophically grown in Petri dishes on medium (Bishop and Senger, 1971) prepared from a stock solution (Table 1) supplemented with 0.5% (w/v) $D^+$-glucose, 0.25% (w/v) yeast extract and agar. Before each experiment, the cultures were transferred from Petri in fresh liquid medium and autotrophically grown for two days into special tubes (~50cm length and diameter Ø 5cm) in a temperature-controlled water bath ($28^0$C), being continuously percolated with air for $CO_2$ supply and also to avoid sedimentation. The illumination

conditions assured during this "prior to UVB irradiation" period were established in function of the scope of UVB irradiation experiments. In general, the *wt* cultures were incubated at a photosynthetically active radiation (PAR, 400-700 nm) intensity of 120 μmoles $m^{-2}s^{-1}$, whereas the *wt-lhc* cultures were maintained at 50 μmoles $m^{-2}$ $s^{-1}$, to avoid any photoinhibitory effect. Light was provided by white fluorescent lamps (36 W Osram, FRG) disposed in a panel placed in the front of the cultures.

**TABLE 1.** Composition of the stock medium used for the autotrophic cultivation of *wt* and *wt-lhc* strains of unicellular green alga *Scenedesmus obliquus*.

| INGREDIENTS | QUANTITY(g/l) | MOLARITY |
|---|---|---|
| $CaCl_2$ x $2H_2O$ | $1.50 \times 10^{-2}$ | $1 \times 10^{-4}$ |
| $KNO_3$ | $80.0 \times 10^{-2}$ | $8 \times 10^{-3}$ |
| $MgSO_4$ x 7 $H_2O$ | $24.6 \times 10^{-2}$ | $1 \times 10^{-3}$ |
| NaCl | $47.0 \times 10^{-2}$ | $8 \times 10^{-3}$ |
| $Na_2HPO_4$ x $2H_2O$ | $17.8 \times 10^{-2}$ | $1 \times 10^{-3}$ |
| $Na_2HPO_4$ x $1H_2O$ | $40.5 \times 10^{-2}$ | $3 \times 10^{-3}$ |
| Na- Citrate x $2H_2O$ | $16.5 \times 10^{-3}$ | $5.5 \times 10^{-4}$ |
| $Fe_2(SO_4)_3$ x $1H_2O$ | $0.40 \times 10^{-3}$ | $7.5 \times 10^{-6}$ |
| $C_6H_5FeO_7$ x $5H_2O$ | $18.025 \times 10^{-3}$ | |
| **Microelements** | | |
| $H_3BO_3$ | $2.86 \times 10^{-3}$ | |
| $MnCl_2$ x $4H_2O$ | $1.81 \times 10^{-3}$ | |
| $ZnSO_4$ x $7H_2O$ | $0.222 \times 10^{-3}$ | |
| $CuSO_4$ x $5H_2O$ | $0.079 \times 10^{-3}$ | |
| $MoO_3$ (85%-99.5%) | $0.0177 \times 10^{-3}$ | |

## 2. EXPERIMENTAL DESIGN

### 2.1. PRELIMINARY MEASUREMENTS

Prior to the development of UVB irradiation experiments, the kinetics of PAR and UVB radiation intensity in the solar light were established by measurements performed during daytime (26 June 2001) at sea level in Heraklion, Crete (Fig. 2). The intensity of PAR (400-700 nm) was measured with QRT1 Quantitherm

lightmeter/thermometer (Hansatech, UK). The intensity of UVB radiation was measured with a VLX-3W radiometer equipped with a CX-312 UVB sensor (Vilber-Lourmat, Cedex, France) and calibrated at 312 nm (accuracy of the measure: ± 5%) with a FL-E lamp by L. N. E. (Laboratoire National d' Essais). Different UVB doses (0.086, 0.137, 0.175, 0.225, 0.317 and 0.420 mW $cm^{-2}$) similar to those found in the solar spectrum were initially tested on the algal cultures (*see* Results and Discussion-*Chapter I*). The maximum quantum yield of PSII, described as Fv/Fm ratio was used as indicator of the culture response to UVB radiation. The strongest effect on Fv/Fm have been obtained with 0.420 mW $cm^{-2}$ UVB intensity and, consequently, this dose was used for UVB treatments of *wt* and *wt-lhc* cultures incubated in different experimental conditions as further described below.

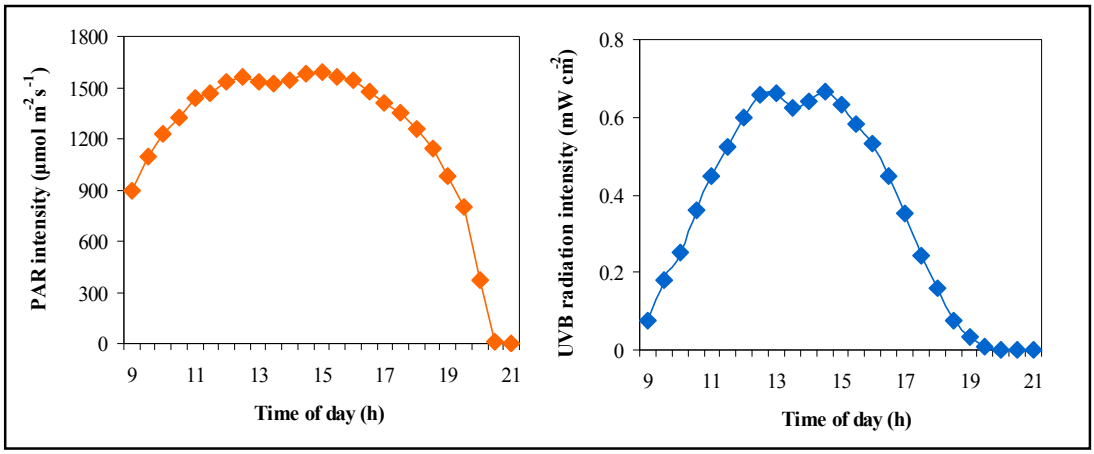


**FIGURE 2.** Kinetics of PAR and UVB radiation intensities in the solar light, as determined by measurements made on a daytime.

## 2.2. EXPERIMENTAL CONDITIONS: UVB TREATMENT AND RECOVERY

Prior to each irradiation experiment, the initial *wt* or *wt-lhc* suspension culture was equally distributed in two cylindrical glass containers (Ø 11.5 cm) forming a layer of 5 cm height, with continuous stirring in a temperature-controlled room (28°C). A VL-6M UVB lamp (Vilbert-Lourmat, Cedex, France) was used as UVB light source being disposed on the top of one of the two cultures. The UVB dose used for treatment was 0.420 mW $cm^{-2}$ at the surface of the culture. The other culture was used as control and covered during UVB irradiation with a plastic UVB filter (Edmund Scientific Company, USA), which cut the UVB radiation below 320 nm.

The UVB treatment was performed for 3 h in different conditions, as described below. For recovery, the cultures were maintained for additional 4 h in the same conditions of PAR illumination.

## 2.3. EXPERIMENTAL CATEGORIES

The experimental categories used in this study are described following the succession of data description used in Results and Discussion.

### 2.3.1. Studying the influence of UVB radiation and PAR intensity conditions on the photosynthetic apparatus response

The *wt* cultures of *S. obliquus* were incubated during experiments in 3 different illumination conditions, namely low PAR (LL, 87 μmol $m^{-2}$ $s^{-1}$) and high PAR (HL, 650 μmol $m^{-2}$ $s^{-1}$) and darkness (D). High PAR intensity was achieved with a Leica P255 slide projector (Germany). A similar procedure consisting in 3 h UVB radiation followed by a 4 h time period for recovery was applied in all cultures. Corresponding control cultures were used for the each variant. The experimental categories were:

a) LL, HL, D = cultures used as controls and maintained in different conditions of illumination as above described;
b) LL+UVB; HL+UVB; D+UVB = cultures from a) category that were treated for 3h with UVB radiation;
c) LL-UVB(R); HL-UVB(R); D-UVB(R) = cultures from b) category after additional 4h of incubation in initial light conditions without UVB.

### 2.3.2. Studying the LHCII role in the photosynthetic apparatus sensitivity to UVB and its regulation by primary photoreceptors

2.3.2.1. Comparative study of the *wt* and *wt-lhc* photosynthetic apparatus responses to UVB and their recovery potential

The *wt* and *wt-lhc* cultures of *S. obliquus* were incubated during 3 h of UVB treatment and additional 4h time period for recovery in low PAR conditions. Corresponding control cultures were used for the each variant. The experimental categories used in this study were:

d) *wt*, *wt-lhc* = cultures used as controls and maintained in different conditions of illumination as above described;

e) *wt*+UVB; *wt-lhc*+UVB = cultures from d) category that were treated for 3h with UVB radiation;

f) *wt*-UVB(R); *wt-lhc*-UVB(R) = cultures from e) category after additional 4h of incubation in conditions without UVB.

2.3.2.2. Action spectra of photosynthetic apparatus sensitivity to UVB

The *wt* and *wt-lhc* cultures were incubated for 24 h in darkness. Prior to UVB irradiation treatments, the cultures were exposed for 3 h in D, white light (WL) or in 20 different wavelengths of monochromatic light (ML) of equal intensity (15 μmol $m^{-2}$ $s^{-1}$). After 3 h of adaptation to different light conditions UVB irradiation experiments were performed for 90 min. After the cessation of UVB treatment, the cultures were let for recovery in the initial D, WL or ML conditions (without UVB radiation) as those applied during UVB treatments. The action spectra were performed with 20 different double interference filters (Edmund Optics) attached to optical fibers of 11 mm diameter connected to 20 M100 (Mille Luce) light sources. Control cultures were used for each variant. The applied 20 wavelengths of monochromatic light, in addition to D and WL, for the construction of action spectra were:

- violet light (400-450 nm): 410, 420, 430, 442 nm;
- blue light (450-500 nm): 455, 470, 492 nm;
- green light (500-550 nm): 510, 535 nm;
- yellow light (550-600 nm): 550, 580 nm;
- orange light (600-650 nm): 600, 620, 632, 640 nm;
- red light (650-700 nm): 650, 656, 671, 690 nm;
- far-red light (>700 nm): 730 nm.

The experimental categories used were:

g) *wt*+ML, *wt*+D, *wt*+WL; *wt-lhc*+ML, *wt-lhc*+D, *wt-lhc*+WL = cultures used as controls, and incubated in different light conditions;

h) *wt*+ML+UVB, *wt*+D+UVB, *wt*+WL+UVB; *wt-lhc*+ML+UVB, *wt-lhc*+D+UVB, *wt-lhc*+WL+UVB = cultures from g) category that were treated for 3h with UVB radiation;

i) *wt*+ML-UVB, *wt*+D-UVB, *wt*+WL-UVB = cultures from h) category after additional 4h of incubation in conditions without UVB (Note: *wt-lhc* cultures did not show recovery, so that they are not mentioned here).

The abbreviations used describe the following:

- *wt*+ML: *wt* strain illuminated with monochromatic light;
- *wt-lhc*+ML: mutant strain illuminated with monochromatic light;
- *wt*+D: *wt* strain incubated in darkness;
- *wt-lhc*+D: mutant strain incubated in darkness;
- *wt*+WL: *wt* strain illuminated with white light;
- *wt-lhc*+WL: mutant strain illuminated with white light.

### 2.3.3. Studying the regulation of photosynthetic apparatus sensitivity to UVB by polyamines

The *wt* and *wt-lhc* cultures were incubated for 2 d in media supplied with 1 mM Put or 1 mM Spm, and then exposed to UVB radiation and recovery in different conditions of illumination.

The experimental categories used for *wt* cultures were:

j) LL, LL+Put, HL, HL+Spm = cultures used as controls and maintained in LL or HL conditions of illumination in the presence or not of exogenously supplied polyamines as above described;

k) LL+UVB, LL+Put+UVB, HL+UVB, HL+Spm+UVB = cultures from j) category that were treated for 3h with UVB radiation;

l) LL-UVB(R), LL+Put-UVB(R), HL-UVB(R), HL+Spm-UVB(R) = cultures from k) category after additional 4h of incubation in conditions without UVB.

The *wt –lhc* cultures treated or not with Put or Spm were incubated in LL conditions. The experimental categories used were:

m) *wt-lhc*, *wt-lhc*+Put, *wt-lhc*+Spm = cultures used as controls, treated or not with 1mM Put or Spm.

n) *wt-lhc*+UVB, *wt-lhc*+Put+UVB, *wt-lhc*+Spm+UVB = cultures from m) category that were treated for 3h with UVB radiation;

o) *wt-lhc*-UVB(R), *wt-lhc*+Put-UVB(R), *wt-lhc*+Spm-UVB(R) = cultures from n) category after additional 4h of incubation in conditions without UVB.

## 3. ISOLATION OF THYLAKOID MEMBRANES

For the preparation of thylakoid membranes the *wt* and *wt-lhc* cultures were centrifuged for 5 min at 1,500 g and the pellets re-suspended in phosphate buffer (pH 7.4). The suspension was mixed with glass beads (Ø 0.2 mm) and broken 4 times for 1 min in a cell mill (Biospec, OK, USA). The homogenate was filtered through a sinter glass filter funnel to separate the glass beads, and centrifuged for 2 min at 500 g to remove unbroken cells and debris. The supernatant was centrifuged for 60 min at 8,000 g. The pellet consisted of two layers. The lower part of the precipitate contained mainly starch and was discarded. The upper, green layer enriched in thylakoid membranes was transferred into a small volume of 0.05 M Tricine buffer (pH 7.4) and used for the polyamine determination and isolation of LHCII sub-complexes (for the *wt* strain) (Sfichi et al., 2004).

## 4. ISOLATION OF LHCII MONOMERIC AND OLIGOMERIC FORMS

For the isolation of LHCII forms, thylakoid membranes, obtained from *wt* cultures as previously described, were subjected to ultra-centrifugation on a continuous sucrose gradient (5-22%), as previously described by Argyroudi-Akoyunoglou and Thomou (1981). For the preparation of sucrose gradient, a buffer was used containing: 0.05 M Tris-HCl, 0.06 M Borate, 0.1% SDS (pH 9.5), in which 0.2% (w/v) deoxycholic acid and 0.2% (w/v) Triton X100 were added. From this buffer were prepared one solution of 5% (w/v) sucrose and another one of 22% sucrose (w/v). Using a peristaltic pump the continuous sucrose gradient was obtained, by mixing 5 mL from the 5% sucrose solution into 5 mL of 22% sucrose, in plastic tubes of 14 mL, suitable for ultracentrifugation (PA, 14x95mm, Nalgene). All procedure took place at $4^{o}$C. To isolate LHCII sub-complexes from isolated thylakoid membranes, the chlorophyll concentration was estimated and a volume of 75mM Tricine-NaOH (pH 7.3) was added so that the final concentration of Chls in the thylakoid sample was 300 μg Chl $mL^{-1}$. The dissolved thylakoids were quickly inoculated at the surface of sucrose gradients and ultracentrifuged in Beckman ultracentrifuge (L8-M, class H) with the SW-40 rotor type (Beckman) at 170 000 × g for 18 h at 4°C in vacuum. After ultracentrifugation, there were obtained in the gradients a superior band of free carotenoids and chlorophylls, and two bands that corresponded to monomeric and oligomeric forms of LHCII (Fig. 3) (Argyroudi-

Akoyunoglou and Thomou, 1981). These bands are fractionated with the peristaltic pump and analyzed for chlorophyll, protein and polyamine content.

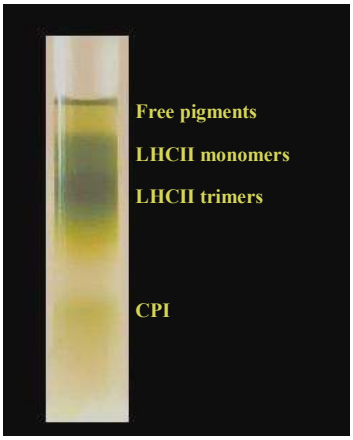


**FIGURE 3.** Separation of the pigment-protein complexes of LHCII from thylakoids isolated from cultures of *Scenedesmus obliquus*, by continuous sucrose density gradient ultracentrifugation (according to method of Argyroudi-Akoyunoglou and Thomou (1981).

## 5. EXTRACTION, QUALITATIVE AND QUANTITATIVE DETERMINATION OF PIGMENTS

Total cell pigments (Chl and Car) were immediately extracted from a standard volume of algal cells by boiling for 1 min in hot methanol, centrifugation of the extract at 1500xg for 5 min and re-extraction of the pellet for 2 additional times with hot methanol until it was colourless. The combined extracts were evaporated to dryness and redissolved in 1 mL of acetone or methanol.

Chl a and b concentrations of the extracts were spectrophotometrically determined (in Perkin Elmer UV/VIS Spectrophotometer) with the extinction coefficients in methanol (Holden, 1965) and calculated by using the equations:

Chl a (μg/mL) = 16.5 (E665) – 8.3 (E650)

Chl b (μg/mL) = 33.8 (E650) – 12.5 (E665)

The concentrations of individual Car were directly determined from the high performance liquid chromatography (HPLC) elution profiles calibrated against standard pigment samples, isolated and purified by thin-layer chromatography (TLC). In this purpose, the pigments from the acetone extract were loaded onto Kieselgel plaques and separated by TLC using a chromatographic medium containing 100mL petroleum ether, 10mL propanol and 250 μL $H_2O$. After separation, each band was isolated and again purified by TLC. The procedure was repeated until all carotenoids were purified. The purified pigments were re-dissolved in different solvents (Britton,

1995) and identified by spectrophotometry in function of their absorption spectrum. The pigments isolated and purified by TLC were: *cis*-neoxanthin, loroxanthin, lutein, violaxanthin, β-carotene, α-carotene, Chl a and Chl b.

*HPLC analysis of pigments*

The quantitative and qualitative determination of carotenes and xanthophylls were made according to the protocol elaborated by Humbeck et al. (1989). Pigment analysis was conducted on the cell extracts obtained as outlined above with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-10AD solvent pumps, an SPD-M10A diode array detector (UV-visible spectrophotometric detector) and a narrow-bore column (C18, 2.1x200 mm, 5 μm particle size Hypersyl, Hewlett-Packard, USA).

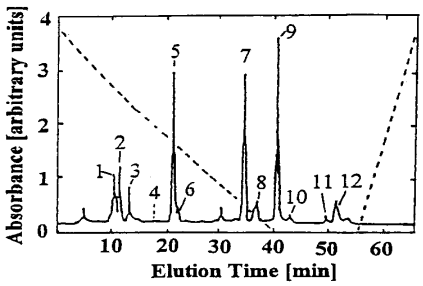


**FIGURE 4.** A typical HPLC elution profile of chlorophylls and carotenoids as obtained from pigment extracts of *Scenedesmus* cultures. The dashed line in the diagram indicates the water gradient superimposed on the solvent system. The numbers in the diagram stand for: **1.** *cis*-neoxanthin (10.5); **2.** trihydroxy-α-carotene (loroxanthin) (11.5); **3.** violaxanthin (13.2); **4.** antheraxanthin (17.8); **5.** lutein (21.5); **6.** zeaxanthin (22.0); **7.** chlorophyll b (34.5); **8.** chlorophyll b' (36.8); **9.** chlorophyll a (40.5); **10.** chlorophyll a' (43.0); **11.** α-carotene (49.5); **12.** β-carotene (51.7). The number in parentheses after the compound name represents their mean elution times. The usual deviation is less than ± 0.2 min. the detection wavelength was 445 nm.

Whole cell extract in methanol (20 μL) was injected onto narrow-bore column; the solvent flow rate was maintained at 0.5 mL $min^{-1}$. The solvent system consisted initially of 85% solvent A (acetonitrile-methanol, 75:25) and 15% solvent B (double distilled water) which, in the first 15 min, was brought to 92.5% solvent A and 7.5% solvent B and then to 100% solvent A over the next 25 min where it was maintained for an additional 20 min. The column was subsequently returned to its original solvent

composition of 85% solvent A and 15% solvent B over the next 11 min prior to the injection of a new sample. Detection wavelength was 445 nm (Fig. 4).

## 6. QUANTITATIVE DETERMINATION OF PROTEINS

The total protein content was determined accordingly to the method of Bradford (1976).

## 7. QUANTITATIVE AND QUALITATIVE DETERMINATION OF POLYAMINES

For polyamine analysis, the samples (pellets, thylakoid membranes, LHCII fractions) were suspended in 1N NaOH and then hydrolyzed according to the procedure of Tiburcio et al. (1985). A volume of 0.2 ml from the hydrolyzate was mixed with 36% HCl in a ratio of 1:1 (v/v) and incubated at 110$^{o}$C for 18 h. The hydrolyzate was evaporated at 70-80$^{o}$C. The dried products were re-dissolved in 0.2 ml of 5 % (v/v) perchloric acid.

To identify and estimate the polyamines, the samples were derived by benzoylation according to the modified method of Flores and Galston (1982), as it is described by Kotzabasis et al. (1993). For this purpose, 1 ml of 2N NaOH and 10 μl benzoylchloride were added to 0.2 ml of the hydrolyzate and the mixture vortexed for 30 s. After 20 min incubation at room temperature, 2 ml of saturated NaCl solution were added to stop the reaction. The benzoylpolyamines were extracted three times into 2-3 ml diethylether; all ether phases collected and evaporated to dryness. The remaining benzoylpolyamines were re-dissolved in 0.2 ml of 63 % (v/v) methanol and 20 μl aliquots of this solution were injected into the high performance liquid chromatography (HPLC) system for the polyamine analysis, as described previously (1993).

The analyses were performed with a Shimadzu Liquid Chromatography apparatus (LC-10AD) equipped with a SPD-M10A diode array detector (Shimadzu SPD-M10A) and a narrow-bore column (C18, 2.1x200 mm, 5 μm particle size Hypersyl, Hewlett-Packard, USA). To directly estimate the amount of each polyamine (Put, Spd and Spm) the method of Kotzabasis et al. (1993) was followed.

## 8. ANALYSIS OF THE PHOTOSYNTHETIC APPARATUS BIOENERGETICS BY USING FLUOROMETRY-BASED ASSAYS

Light energy that is absorbed by chlorophylls can undergo three fates: a) it can be used to drive photosynthesis (photochemistry), b) it can be dissipated as heat or c) it can be re-emitted as red fluorescence (Fig. 5). These three processes occur in competition. Since their sum is a constant, any increase in the efficiency of one process will result in a decrease in the yield of the other two. Therefore, measurements of the Chl fluorescence yield will give information about changes in the efficiency of photochemistry and heat dissipation.

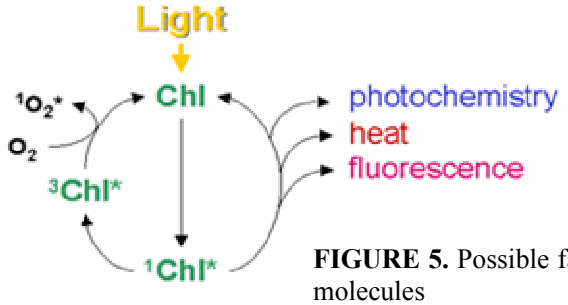


**FIGURE 5.** Possible fates of excited chlorophyll molecules

### 8.1. ANALYSIS OF MODULATED FLUORESCENCE BY THE SATURATION PULSE METHOD

A typical measurement is shown in Figure 6. The culture sample is dark-adapted for 10 min (depending on temperature) prior to measurements of Chl a fluorescence using a PAM (Pulse Amplitude Modulated) fluorometer (Heinz Walz, Germany) The fluorescence $F_0$ is measured by using a modulated light (ML) with a low intensity (1 μmol $m^{-2}$ $s^{-1}$) to avoid the reduction of the PSII primary electron acceptor, $Q_A$. The maximal fluorescence yield, Fm, is induced by a short saturating pulse (SP) of white light (3650 μmol $m^{-2}$ $s^{-1}$ intensity, 0.4 s duration), which triggers the reduction of all $Q_A$. The fluorescence yield Fs reflects the electron transport under actinic light (AL) (110 μmol of photons $m^{-2}$ $s^{-1}$). The maximal fluorescence yield in the light-adapted state Fm' is induced by SP given periodically at every 30 s when alga is exposed to continuous AL. At the steady state of electron transport, AL is turned off and a far-red light (FR) is applied to ensure rapid oxidation of $Q_A$. The fluorescence $F_0$' for light-adapted sample represents the fluorescence yield, when all

PSII reaction centers are in open state. At steady state of fluorescence yield (6 min under continuous actinic light), the difference between Fm' and Fs reflects the photochemical part of fluorescence quenching which is expressed in the *operational quantum yield of PSII* as: $\Phi s_{PSII}$ = (Fm'-Fs)/Fm' (Genty et al., 1989).

The PSII quantum efficiency is affected by the level of electron acceptors, usually NADP+, available at the acceptor side of PSI. Consequently, $\Phi s_{PSII}$ decreases in situations with limiting consumption of NADPH like for example at low internal $CO_2$ concentration. Changes in $\Phi s_{PSII}$ can be attributable to differences in the capacity for electron flux on the reducing side of PSII, as mentioned above, but also to down-regulation of PSII. The PSII quantum efficiency frequently exhibits a strong, quantitative relationship with the quantum yield of $CO_2$ assimilation. Multiplied with the amount of absorbed light by PSII, $\Phi s_{PSII}$ is a measure of the rate of linear electron transport through photosystem II.

*The electron transport rate* (ETR) can be calculated as: ETR = $\Phi s_{PSII}$ x PPFD x 0.5, where PPFD is the photosynthetic photon flux density (e.g. 110 µmol quanta $m^{-2}$ $s^{-1}$) and 0.5 is a factor that accounts for the portioning of energy between PSII and PSI. The maximum ETR is achieved when the capacity of the sum of all electron sinks (carbon fixation, photorespiration, nitrate assimilation, Mehler reaction) is reached.

*The non-photochemical quenching* (qN) described the level of non-radiative energy dissipation in the light-harvesting antenna of PSII. The non-photochemical quenching prevents the over-reduction of the electron transfer chain and, therefore, provides protection from photodamage. The most straightforward way to quantify non-photochemical quenching (qN) is by measuring the fluorescence parameters, using the equation: qN= 1- ((Fm'-$F_0$')/(Fm-$F_0$)).

In higher plants, non-photochemical quenching can be divided into at least two different components according to their relaxation kinetics. The most rapidly relaxing component is the ΔpH- or energy-dependent non-photochemical quenching, often named qE, which relaxes within seconds to minutes. The level of qE is dependent upon both the ΔpH, which is essential and zeaxanthin content that acts as a positive effector. The second component of qN is qT, which relaxes within minutes and is more important in algae, but rather negligible in most plants during exposure to excess light. This component is due to the phenomenon of state transition, the

uncoupling of LHCII from PSII. The remaining part of qN that relaxation is markedly slower than that of qE is called photoinhibitory non-photochemical quenching, qI. Increasing proportion of qI on qN is an indicator for enhanced stress.

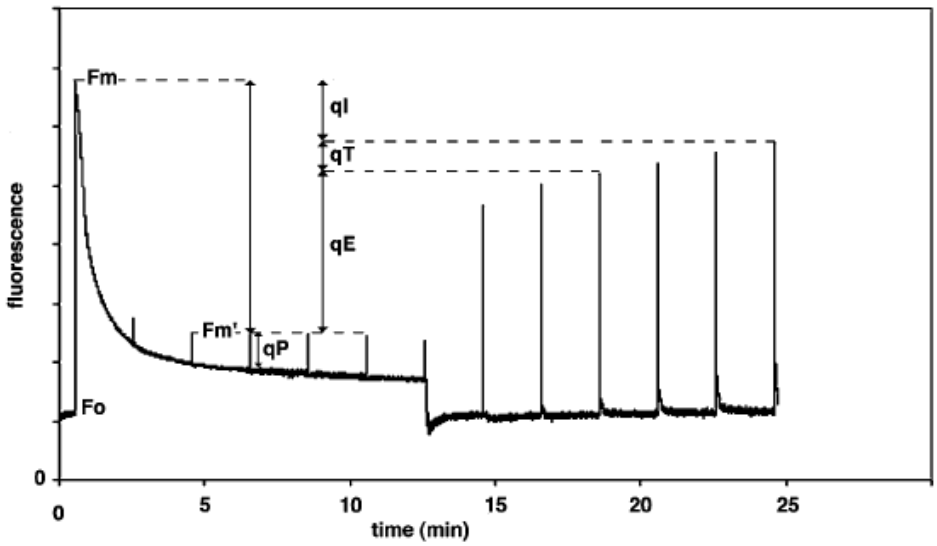


**FIGURE 6.** Typical Chl fluorescence measurements for the investigation of photochemical and non-photochemical quenching parameters by PAM fluorometry

*Photochemical quenching* (qP) value gives an indication of the PSII reaction centers that are open and it is calculated as: qP = ((Fm'-Fs)/(Fm'-$F_0$')). An alternative expression of this is (1-qP), representing the proportion of centers that are closed and it is referred as the *excitation pressure* of PSII (Maxwell and Johnson, 2000).

## 8.2. HIGH RESOLUTION ANALYSIS OF FAST FLUORESCENCE O-J-I-P TRANSIENTS

The polyphasic fluorescence rise from $F_0$ (O level) via J-I phase to Fm (P level) of a Kautsky curve (Fig. 7) (Strasser et al., 1995) is widely accepted to reflect the accumulation of the reduced form of the primary quinone acceptor $Q_A$ (i.e. the reaction center closure), which is the net result of $Q_A$ reduction due to PSII activity and $Q_A^-$ reoxidation due to PSI activity. It is assumed that under normal conditions $Q_A$ is completely oxidized in the dark, i.e. all reaction centers are open, and the fluorescence signal at the onset of illumination is $F_0$. The maximum yield Fp depends

on the achieved reduction-oxidation balance and acquires its maximum possible value, Fm, if the illumination is strong enough (above 100 W $m^{-2}$) to ensure the closure of all reaction centers. Transients were recorded with high time-resolution fluorimeters (PEA, Plant Efficiency Analyzer; Hansatech Ltd., King's Lynn, Norfolk, UK) using an actinic light intensity of 600 $Wm^{-2}$. The actinic light of the PEA is provided by an array of six light emitting diodes (emission around 650 nm) which are focused on the sample surface. Chlorophyll a fluorescence is detected by a photodiode located behind a long pass-filter (50% transmission at 720 nm).

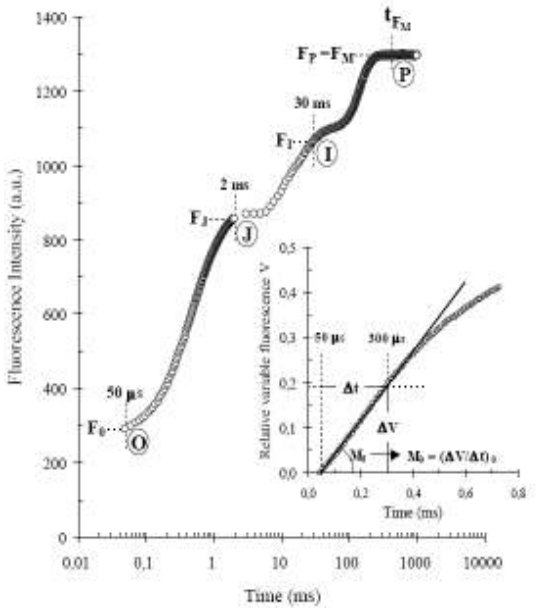


**FIGURE 7.** A typical Kautsky fluorescence transient exhibited upon illumination of a dark-adapted photosynthetic sample by saturating light (red light of 600 $Wm^{-2}$) and plotted on a logarithmic time scale from 50 µs to 1 s. The marks refer to the selected fluorescence data used by the JIP-test for the calculation of structural and functional parameters. The signals are: the fluorescence intensity $F_0$ (at 50 µs); the fluorescence intensities $F_J$ (at 2 ms) and $F_I$ (at 30 ms); the maximal fluorescence intensity, $F_P$ = Fm (at $t_{Fm}$). The inset presents the transient expressed as the relative variable fluorescence V = $(F-F_0)/(Fm-F_0)$ vs. time, from 50 µs to 1 ms on a linear time scale (Strasser et al., 2000).

Chl a fluorescence signals were recorded in a time span ranging from 10 µs to 10 s with a minimal data acquisition interval of 10 µs (12 bit resolution). The light intensity of the LEDs at 50 µs had reached more than 99% of its final value and the response time of the fluorescence detector is clearly smaller than 50 µs. The fluorescence signal at 50 µs was considered to be a true $F_0$ because the fluorescence yield at this time was found to be completely independent of the actinic light intensity. The fluorescence yield at $F_m$ was clearly saturated since a 75% decrease in light intensity did not change the fluorescence yield. All experiments were performed with 1000 µL of cell suspension in 1 cm diameter vials. The thickness of the sample was

approximately 6 mm and the diameter of the irradiated sample area was 1 cm. Before measurement of fluorescence transients cells were dark-adapted for 10 min. Far-red pre-illumination (5 min) after dark adaptation did not induce any changes in the fluorescence transients. It can be conclude that the photosynthetic apparatus is in State 1 with the PQ pool in its oxidized state.

The investigation of fluorescence transients was also performed in the presence of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which blocks the electron transfer from $Q_A^-$ to $Q_B$ by its binding in the $Q_B$ niche of D1 protein of PSII reaction centers. For measurements on DCMU-inhibited samples (Trebst, 1980), DCMU was dissolved in ethanol and then, in complete darkness, mixed with the sample 10 min prior to the measurement. The final DCMU concentration was 100 μM; the final ethanol content was below 0.1% (v/v).

*JIP-test analysis*

A quantitative analysis of the O-J-I-P transient has been introduced (Strasser and Strasser, 1995) and further developed (for a review see Strasser et al, 2000), named as the "JIP-test" after the basic steps of the transient, by which several phenomenological and biophysical - structural and functional - parameters quantifying the PSII behaviour are calculated. The JIP-test was proven to be a very useful tool for the *in vivo* investigation of the adaptive behaviour of the photosynthetic apparatus and, especially, of PSII to a wide variety and combination of stressors, as it translates the shape changes of the O-J-I-P transient (Fig. 7) to quantitative changes of the several parameters (Table 2). The JIP-test provides adequate information about the behaviour (structure, conformation and function) of the photosynthetic apparatus being at any physiological state.

## 8.3. DETERMINATION OF THE FUNCTIONAL PSII-HETEROGENEITY

The redox state of $Q_A$, the primary quinone acceptor of PSII, is determined by its photochemical reduction due to PSII activity and its re-oxidation by the electron transport driven by PSI activity. In order to reduce the complexity of the *in vivo* system and facilitate the investigation of PSII properties, the utilization of diuron (DCMU), has been widely employed. In the presence of DCMU at room temperature,

**TABLE 2.** Summary of parameters, formulae and their description using data extracted from the fast fluorescence O-J-I-P transients.

| | |
|---|---|
| **FLUORESCENCE PARAMETERS** | |
| $F_o$ | Fluorescence intensity at 50 μs |
| $F_{100\ \mu s}$ | Fluorescence intensity at 100 μs |
| $F_{300\ \mu s}$ | Fluorescence intensity at 300 μs |
| $F_J$ | Fluorescence intensity at 2 ms |
| $F_I$ | Fluorescence intensity at 30 ms |
| $F_m$ | Maximal fluorescence intensity |
| $V_J$ | Variable fluorescence at 2 ms;<br>$[(F_J\text{-}Fo)/(Fm\text{-}Fo)]$ |
| $M_o$ | The rate of reaction center closure;<br>$[4 \times ( F_{300\ \mu s} - Fo) / ( Fm - F_o )]$ |
| **QUANTUM EFFICIENCIES OR FLUX RATIOS** | |
| *Fv/Fm* or $\Phi_{Po}$ | Maximum quantum yield of PSII;<br>[(Fm-Fo)/Fm] |
| *PSIo* or $\Psi_o$ | Efficiency with which an exciton can move an electron further than $Q_A^-$ in the electron transport chain;<br>$[1- V_J]$ |
| **SPECIFIC FLUXES OR SPECIFIC ACTIVITIES** | |
| *ABS/RC* | Absorbance per active reaction center - a measure for the functional antenna size;<br>$[Mo \times (1/V_J) \times (1/\Phi_{Po})]$ |
| *TRo/RC* | Efficiency of exciton trapping per reaction center;<br>$[Mo \times (1/V_J)]$ |
| *DIo/RC* | Rate of energy dissipation per active reaction center;<br>[ABS/RC - TRo/RC] |
| **DENSITY OF REACTION CENTERS** | |
| *RC/CS* | The density of active reaction centers per cross section;<br>$[\Phi_{Po} \times (V_J/Mo) \times Fm]$ |
| **PERFORMANCE INDEX** | |
| *PI(abs)* | It is a measure for the primary photosynthetic performance;<br>$[RC/ABS \times (\Phi Po /(1\text{-}\Phi_{Po}) \times (\Psi_o/(1\text{-}\Psi_o))]$ |
| **OVERALL GROUPING PROBABILITY** | |
| *pG* | It is a measure to estimate the excitation energy transfer among PSII;<br>$[(W_{E,\ 100\mu s}\text{-}W_{100\mu s})/( W_{100\mu s} \times (1\text{-} W_{E,\ 100\mu s} \times V_J) \times V_J) \times (Fo/(Fm\text{-}Fo))]$, where<br>$[W_{E,\ 100\mu s}= 1\text{-}(1\text{-}((F_{300\mu s}\text{-}Fo)/(F_{2ms}\text{-}Fo))^{1/5}]$, $[W_{100\mu s}= (F_{100\ \mu s}\text{-}Fo)/(F_{2ms}\text{-}Fo)]$ |
| **QUENCHING PARAMETERS** | |
| *qPQ* | Fluorescence parameter expressing quenching due to oxidized PQ;<br>$[(Fm\text{-}F_{30\ ms})/(Fm\text{-}F_0)]$ |
| *qEmax* | Capacity for high-energy non- photochemical quenching;<br>$[Fm\text{-}F_{6sec})/Fm]$ |

the fluorescence kinetics reflect pure photochemical events leading to the complete reduction of $Q_A$ (full closure of reaction centers).

The area over the fluorescence induction kinetics increases with first order kinetics (Melis and Homann, 1975, 1976). It is a measure of the number of quanta not emitted as fluorescence and used in photochemistry. Two possibilities are considered to explain the biphasic nature of the area growth. The first is a sequential double reduction of the primary electron acceptor in PSII, while the second envisages heterogeneity of its photochemical centers. Assuming the existence of a heterogeneous pool of photochemical centers, the growth of the area over the fluorescence curve could be further analyzed to yield two components, a fast α-component, and a relatively slow β-component.

The kinetic characteristics of these components, and the effect of a short saturating flash on their respective size, led to the conclusion that one type of photochemical center has a faster recombination rate of the photochemically separated charges and was less efficient in trapping excitation energy. The parameter ln((AREA-AREAt)/AREA) is used to make the distinction between the two components. The fast component corresponds to PSII-α reaction centers with bigger LHCII antenna, considered to be located in the grana fraction. The slow component is corresponding to PSII-β reaction centers, which possess a smaller LHCII antenna, considered to be situated in the stromal thylakoids (Melis, 1989). Using PEA, the fluorescence induction curves in DCMU-poisoned samples were analyzed and the $K_\alpha$ and $K_\beta$ constants for the each population of PSII were determined according to Melis and Homann (1975, 1976). In addition, the $Q_B$ non-reducing centers were also calculated using the formula: $V_{J\ (without\ DCMU)}/F_{v\ (+DCMU)}$ (Guenther and Melis, 1990).

## 9. POLAROGRAPHIC MEASUREMENTS

Maximal net photosynthetic rate (oxygen evolution) was calculated from the maximal photosynthetic rate and maximal respiratory rate and expressed as μmol $O_2$ mg $Chl^{-1}$ $min^{-1}$. Both parameters were determined polarographically at $30^oC$ with a Clark type electrode system (Hansatech Instruments, Kings's Lynn, Norfolk, UK). The algal pellet collected by centrifugation at 1500xg of suspension cultures with the density adjusted to 10μl PCV/mL was dissolved in 10mM Tricine (pH 7.6) to give a final volume of 2mL and loaded into the cuvette of system. The actinic light (470 W

$m^{-2}$) was generated with two lamps (ENX360W/82V) and its intensity measured with a sensitive photoradiometer (International Light, Newburyport, MA, 01950) consisting of a control box (IL 1700), a power supply (IL 760) and a photomultiplier (IL 780). The infrared part of the applied irradiation was filtered off by inserting a 2 % $CuSO_4$–containing cuvette (2 cm path length) into the light beam.

## 10. DETERMINATION OF ROS ACCUMULATION.

### 10.1. *In vivo* DETECTION OF ROS USING DCFH-DA

ROS production was detected by using dichlorofluorescin diacetate (DCFH-DA). This non-polar compound is converted to the polar derivative DCFH by cellular esterases when it is taken up. DCFH is nonfluorescent but highly fluorescent when oxidized to dichlorofluorescein (DCF) by intracellular ROS and other peroxides (He and Häder, 2002). DCFH-DA cannot be added or incubated prior to UV or PAR irradiation since it is rapidly autooxidized. DCFH is hydroliyzed by UV radiation, but much slower by ambient light and in the dark, especially in aqueous solution. In addition, the production of the fluorescent DCF is affected by the incubation temperature. Therefore, it is important to maintain constant incubation conditions, including temperature and minimal ambient light. DCFH- DA (final concentration 5 μM) was immediately added to the irradiated culture and incubated on a shaker at room temperature in the dark for 1 h. The fluorescence of the samples was measured with a spectrofluorometer (RF-5000, Shimadzu, Kyoto, Japan) at room temperature, by an excitation of 485 nm and an emission band between 500 and 600 nm. The fluorescence intensity at 520 nm normalized to the protein content was used to determine the relative ROS production.

### 10.2. DETECTION OF ROS IN ISOLATED THYLAKOID MEMBRANES USING CHEMILUMINESCENCE ASSAY

#### 10.2.1. Luminol- dependent chemiluminescence assay for hydrogen peroxide ($H_2O_2$)

The production of $H_2O_2$ in thylakoid membranes was estimated in 1 mL of thylakoid suspension, isolated as previously described. The assay was conducted in a total volume of 2 mL by placing 0.8 mL of reaction buffer containing 10mM Tris-Mes (pH 7.0), 1mM $CaCl_2$, 0.1 mM KCl, 0.2 mL of 1mM luminol solution, 0.1 unit of

peroxidase in 20 mM potassium phosphate buffer (pH 7.4) and 1 mL of thylakoid membranes in a scintillation vial (Auh and Murphy, 1995). The vial was immediately placed in a scintillation spectrometer (model LS 8000, Beckman) and chemiluminescence was detected. Counts were reported every 15 s for 1 min and the last two values were averaged. Data obtained were reported to a standard curve, which correlates the chemiluminescence values to standard concentrations of $H_2O_2$. Using the analogy 106 cpm=765.3 ±21.4 pmol $H_2O_2$, the production of $H_2O_2$ was calculated.

### 10.2.2. Lucigenin-dependent chemiluminescence assay for superoxide ($O_2^{\cdot-}$)

The accumulation of superoxide was measured by the chemiluminescence of lucigenin, which is specific for $O_2^{\cdot-}$ (Corbisier et al., 1987) in thylakoid membranes isolated as previously described. The assay was conducted in a total volume of 2 mL by placing 0.2 mL of 1 mM lucigenin solution, 0.8 mL of 0.1 M Gly-NaOH buffer (pH 9.0) containing 1 mM EDTA and 1 mL of thylakoid suspension (Auh and Murphy, 1995). All of the other conditions described for $H_2O_2$ assays were followed. Counts were reported every 6 s for 30 s, and the last two values were averaged. In accordance to xanthine/xanthine oxidase system (Murphy and Auh, 1996), 106 cpm correspond to 33.14 ± 2.1 pmol $O_2^{\cdot-}$. Using this analogy, $O_2^{\cdot-}$ production was calculated.

## 11. DETERMINATION OF THE PACKED CELL VOLUME (PCV)

The PCV of a cell suspension was determined by centrifugation at 1500xg for 5 min using haematocrite tubes (Senger et al., 1993) and was expressed as µL PCV/mL culture.

## 12. STATISTICAL ANALYSIS

All experiments were performed at least three times and for each repetition 3 to 5 samples were measured. The values given for each parameter represent the average of the values obtained for each repetition. The standard deviations were also calculated and represented in the afferent diagrams.

# RESULTS AND DISCUSSION

# *CHAPTER I*

## MODULATION OF UVB EFFECTS IN THE PHOTOSYNTHETIC APPARATUS BY PAR

## *RESULTS*

The exposure of plants to light energy in excess of that required for photosynthesis results in an energy imbalance between the energy absorbed through the light harvesting complex and the energy that can be dissipated or transduced by PSII and PSI, which generally leads to photoinhibition. Such imbalance may be generated by high light alone or may be enhanced by biotic and abiotic stress factors resulting in excess excitation energy (Strid et al., 1994; Huner et al., 1998; Karpinski et al., 1999). Many studies have identified PSII as the most labile component of the photosynthetic apparatus to elevated UVB radiation (Iwanzik et al., 1983; Strid et al., 1990; Melis et al., 1992; Vass et al., 1996; Mackerness et al., 1997; Jansen et al., 1998), but the underlying mechanisms are still a controversial subject (Anderson and Aro, 1994) and this makes it difficult to evaluate the environmental relevance of UVB effects on photosynthesis.

The response of a plant to UVB radiation is the net result of damaging reactions, repair and acclimation responses (Jansen et al., 1999). Whether UVB radiation is a stress factor depends on wavelength, irradiance and exposure time, as well as on the genetic, morphological and protective predisposition of the plant species or cultivar. Indeed, two likely reasons underlying the discrepancy between UVB studies are the unnatural amplification of damaging reactions as a result of the excessive UVB fluence rates used and a failure to take into consideration naturally occurring tolerance mechanisms (Fiscus and Booker, 1995; Rozema et al., 1997; Jansen et al., 1998). In addition to unrealistically high UVB irradiance used, another criticism of many previous studies has been the low photosynthetically active radiation (PAR: 400-700 nm) under which the plants were grown and irradiated. In only few studies were investigated the effects of UVB radiation on plants grown under relatively high PAR, which approximate the natural conditions (Mirecki and Teramura, 1984; Bornman and Vogelman, 1991; Flores-Moya et al., 1999; Krause et al., 1999).

In this context this study focuses on UVB effects on the conformation, structure and function of the photosynthetic apparatus in order to distinguish between damage and protective mechanisms to UVB. The experiments were designed so as to make possible the investigation of the sensitivity/adaptation mechanisms to UVB radiation and the modulatory role of PAR without exacerbating the UVB effect. To achieve this it was necessary to establish a UVB dose able to induce a strong and rapid stress response to the photosynthetic apparatus without affecting the cell capacity for recovery. After the determination of daily PAR/UVB ratio by measurements of UVB and PAR intensities in the solar light (*see* Fig. 2-Material and Methods), different UVB fluences ranging from 0.087 to 0.420 mW $cm^{-2}$ were initially tested for their effect on the PSII functionality in *Scenedesmus obliquus* cultures exposed to a PAR intensity of 87 µmol $m^{-2}$ $s^{-1}$ (Fig. 1).

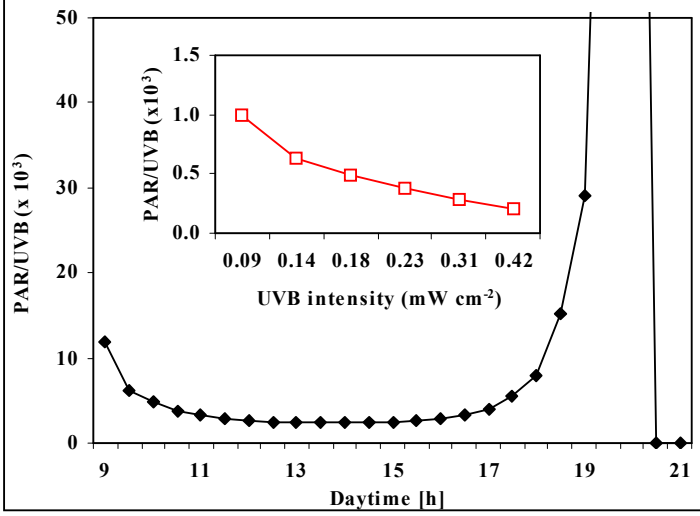


**FIGURE 1.** PAR/UVB ratio kinetics in the solar light, determined from the measurements of PAR and UVB intensities (*see* Fig. 2, Material and Methods 2.1.). The inset show the values of PAR/UVB ratio for the UVB doses tested in algal cultures exposed to 87 µmol $m^{-2}$ $s^{-1}$.

The results show that UVB affected the PSII functionality, estimated as the maximum quantum yield of PSII (Fv/Fm) and the density of active reaction centers (RC/CS) in a time/dose dependent manner (Fig. 2A-B). The UVB effect is absent below 0.100 mW $cm^{-2}$, while at higher doses the response seems to be a mixture of time-dependent and time-independent behavior. The early part of the exposure can be described as cumulative fluence (and is, therefore, time-dependent/dose-independent), while the latter phase seems to be a function of fluence-rate (time-independent/dose-dependent). This is better expressed in Figure 2C**,** where the exponential decrease in PSII photochemistry related to the inactivation of reaction centers becomes more

accentuated after 3 h of UVB irradiation. The highest effect (about 65% Fv/Fm decrease) was obtained for 0.42 mW $cm^{-2}$ UVB and consequently this dose was applied in all further experiments for the study of UVB effects on the photosynthetic apparatus. This was made in relation to the influence of PAR, since it is well known that visible light intensity influences the response of the photosynthetic apparatus to UVB radiation. The dose of 0.42 mW $cm^{-2}$ UVB was applied in low light (LL, 87 µmol $m^{-2}$ $s^{-1}$) and high light conditions (HL, 650 µmol $m^{-2}$ $s^{-1}$), which means that in LL conditions the ratio PAR/UVB is almost 11 times lower than the natural measured one, while in HL this ratio is approximately at the level estimated in the solar light at 13.00 in the day (*see* Fig. 2, Material and Methods 2.1.). In this way, the investigation of UVB effects could give a measure of the amplitude of damage/protection degree in natural irradiance conditions, as well as, in a fictive scenario of UVB increasing due to ozone depletion.

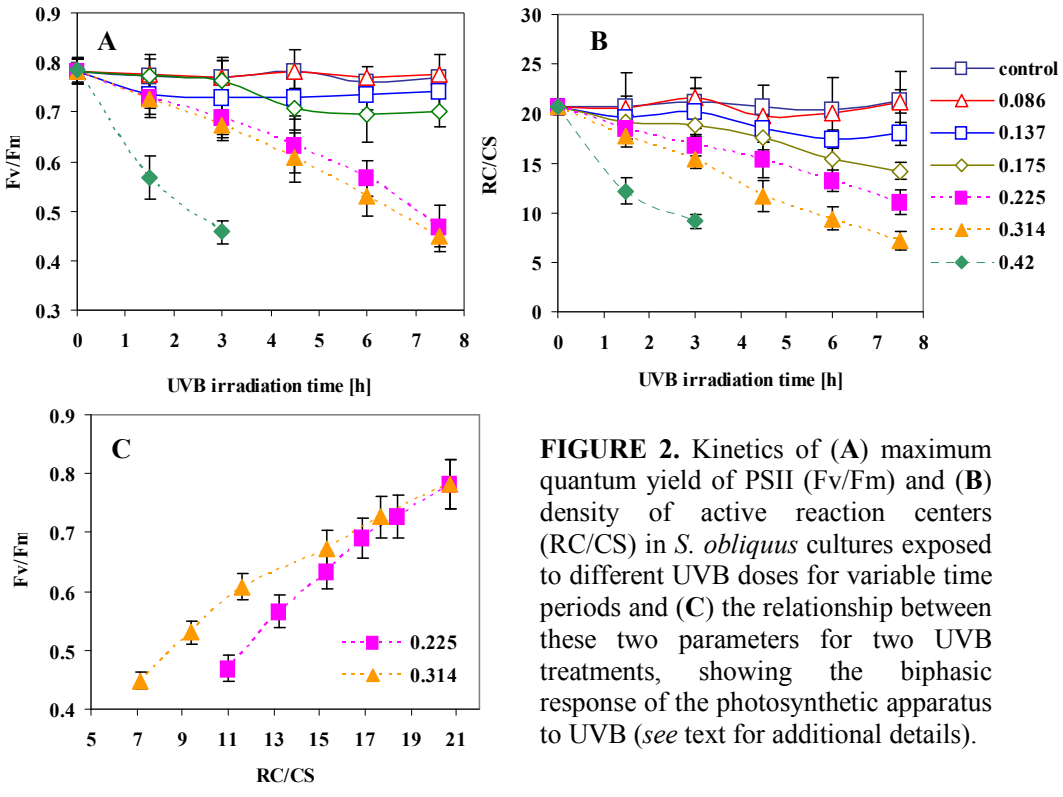


**FIGURE 2.** Kinetics of (**A**) maximum quantum yield of PSII (Fv/Fm) and (**B**) density of active reaction centers (RC/CS) in *S. obliquus* cultures exposed to different UVB doses for variable time periods and (**C**) the relationship between these two parameters for two UVB treatments, showing the biphasic response of the photosynthetic apparatus to UVB (*see* text for additional details).

To estimate the importance of PAR in the modulation of photosynthetic apparatus response to UVB, algal cultures were also exposed to UVB in complete

darkness (D). A similar protocol consisting from 3 h of UVB irradiation treatment and additional 4 h of incubation for recovery (after the cessation of UVB treatment) was applied in each experimental category (*see* Material and Methods 2.3.1.).

## 1. CHANGES IN PHOTOSYNTHETIC APPARATUS BIOENERGETICS UPON UVB IRRADIATION

The processing of light energy through PSII was investigated by means of recording the transients of Chl a fluorescence and using the JIP-test and associated parameters (Strasser and Strasser, 1995; Strasser et al. 1995; Krüger et al. 1997; Strasser et al. 2000).

### 1.1. THE O-J-I-P TRANSIENTS

It is generally accepted that fluorescence induction curves, reflecting the photosynthetic activity and electron transport, have a characteristic pattern that undergo changes when the photosynthetic systems are impaired and can be used as an indicator of damage (Strasser et al., 2000). Therefore, the polyphasic kinetics of the Chl fluorescence rise from $F_0$ (O level) via J-P phase to Fm (P level) of a Kautsky curve (Strasser et al., 1995) were examined at three experimental stages: prior to UVB irradiation (control, C), after 3 h of UVB irradiation (UVB) and after 4 h of recovery (R).

The shape of O-J-I-P transients showed changes upon UVB irradiation, which are intensified or diminished in relation to the PAR intensity used as background for UVB treatment. Specifically, UVB induced the reduction of Fm (P peak) and of I-P phase which, in LL+UVB and D+UVB cultures, culminated with the disappearance of I step and was accompanied by an increase in $F_0$ and, in general, by a rise in the fluorescence between 50 μs and 2 ms (O-J phase) (Fig. 3). These changes in Chl fluorescence recovered after the cessation of UVB treatment only in the cultures incubated in light conditions (LL-UVB(R) and HL-UVB(R) cultures). Recovery did not occur in D, where the functionality of PSII was irreversibly damaged by UVB. These results highlight the importance of visible light (PAR) intensity for the development of repair processes, as well as, for the determination of damage amplitude. From the data obtained by measurements of Chl fluorescence, several important parameters for the estimation of structure, conformation and function of the photosynthetic apparatus were determined.

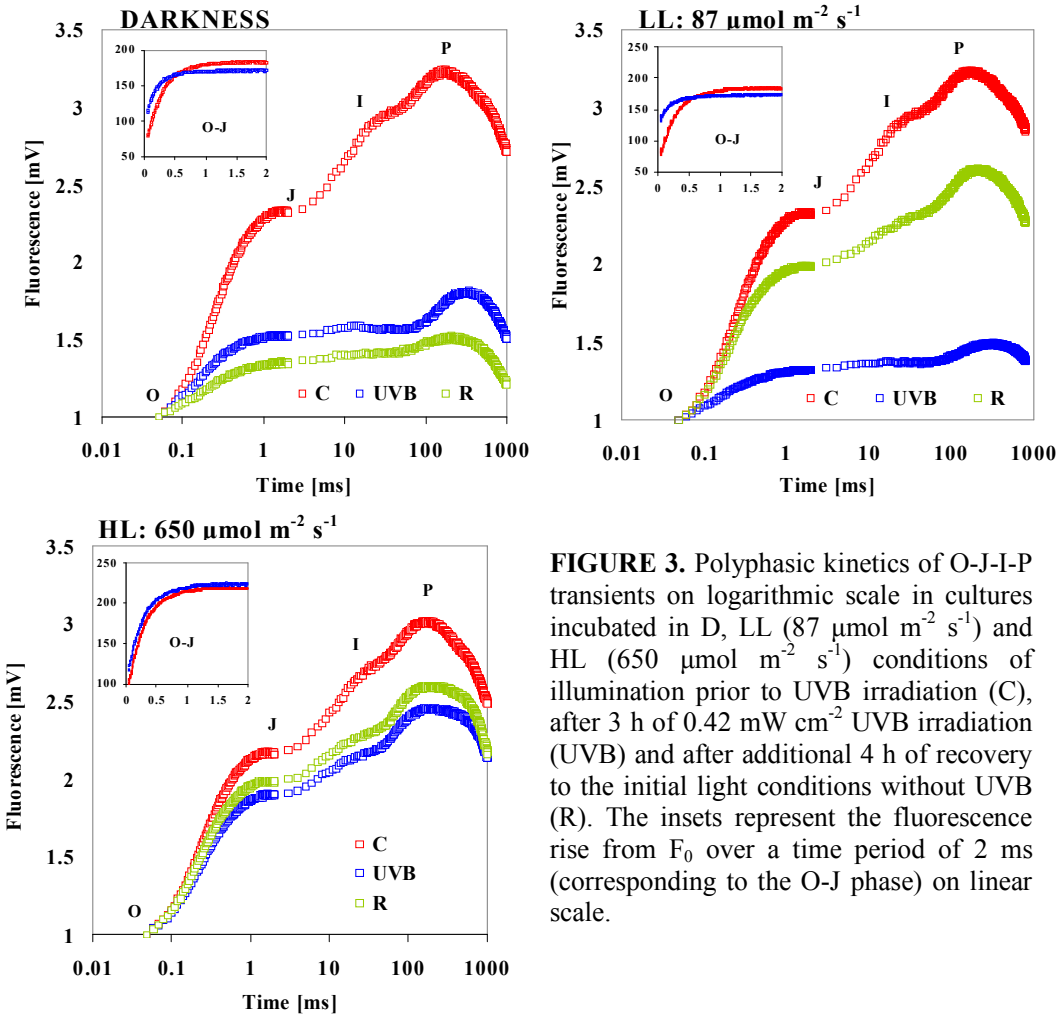


**FIGURE 3.** Polyphasic kinetics of O-J-I-P transients on logarithmic scale in cultures incubated in D, LL (87 µmol $m^{-2}$ $s^{-1}$) and HL (650 µmol $m^{-2}$ $s^{-1}$) conditions of illumination prior to UVB irradiation (C), after 3 h of 0.42 mW $cm^{-2}$ UVB irradiation (UVB) and after additional 4 h of recovery to the initial light conditions without UVB (R). The insets represent the fluorescence rise from $F_0$ over a time period of 2 ms (corresponding to the O-J phase) on linear scale.

## 1.2. PSII FUNCTIONALITY UPON UVB TREATMENT

The decrease in Fm accompanied by an increase in $F_0$ in LL+UVB and D+UVB cultures resulted in a decrease in the variable fluorescence Fv (Fig. 4A) and subsequent changes in the Fv/Fm, $F_0$/Fv and Fv/$F_0$ parameters (Fig. 4B). These are widely used in literature as a measure to estimate stress effect on PSII photochemistry. As shown in Figure 4B, both Fv/Fm and Fv/$F_0$ decreased upon UVB, whilst $F_0$/Fv is increasing. The intensity of such changes was highest in LL+UVB and D+UVB cultures, where $F_0$ registered a pronounced increase. Due to this effect, the maximum quantum yield of overall PSII photochemistry (Fv/Fm) decreased about 65% in LL+UVB conditions. In contrast, the decrease of Fv/Fm was about 35% in HL+UVB culture (Fig. 5A).

The JIP test and associated parameters have been used to study stress effects related to high temperature (Srivastava et al., 1997), high light (Krüger et al. 1997), ozone (Navakoudis et al., 2003) but less used in UVB studies. Using the JIP-test (Strasser and Strasser, 1995) in this study, several expressions were used for estimation of the structure, conformation and function of the photosynthetic apparatus; they were calculated and the results are described below.

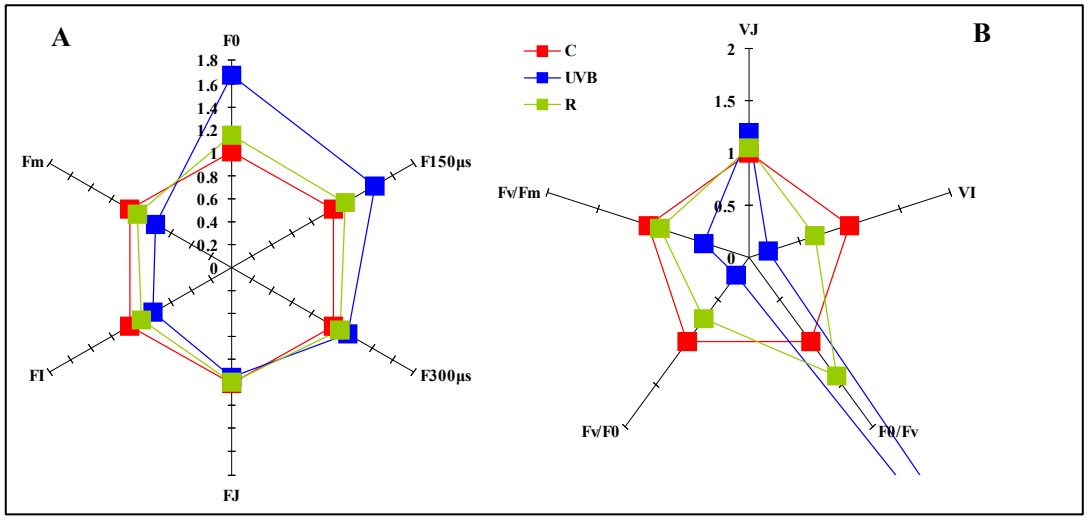


**FIGURE 4.** Radar plots of fluorescence parameters after UVB treatment (UVB) and recovery (R) as compared to control (C). The amplitude of changes, upon UVB irradiation, in chlorophyll fluorescence at different points of electron transfer chain (**A**) resulted in the modification of variable fluorescence at 2ms ($V_J$) and 30 ms ($V_I$) and of several parameters (Fv/Fm, $F_0$/Fv, Fv/$F_0$) commonly used to describe the PSII functionality (**B**).

The absorbance per reaction center (ABS/RC) expresses the total absorption of PSII antenna chlorophylls divided by the number of active (in the sense of $Q_B$ reducing) reaction centers. Consequently, this flux may be regarded as a measure of light harvesting complex (antenna) size (Strasser and Strasser, 1995). In Figure 5C can be seen that, comparative to control cultures, the functional antenna size was clearly increased by UVB irradiation. This effect was stronger in LL+UVB culture, comparative to those incubated in HL+UVB or D+UVB. After the cessation of UVB treatment, the functional antenna size decreased, gradually, to a level closely similar to that of control. Recovery occurred only in light-incubated cultures and was more pronounced in LL-UVB(R) than in HL-UVB(R) conditions. In darkness, the size of functional antenna continued to increase, even after 2h from the cessation of UVB treatment. It has been suggested that an increase in functional antenna size can result

from the increase of the number of chlorophyll per reaction center, the inactivation of some reaction centers or the modification of the rate constants for any excitation energy transfer (Strasser et al., 2000). The results in Figure 5 indicate that at least two of these mechanisms (e.g. the last ones) were responsible for increase in the functional.

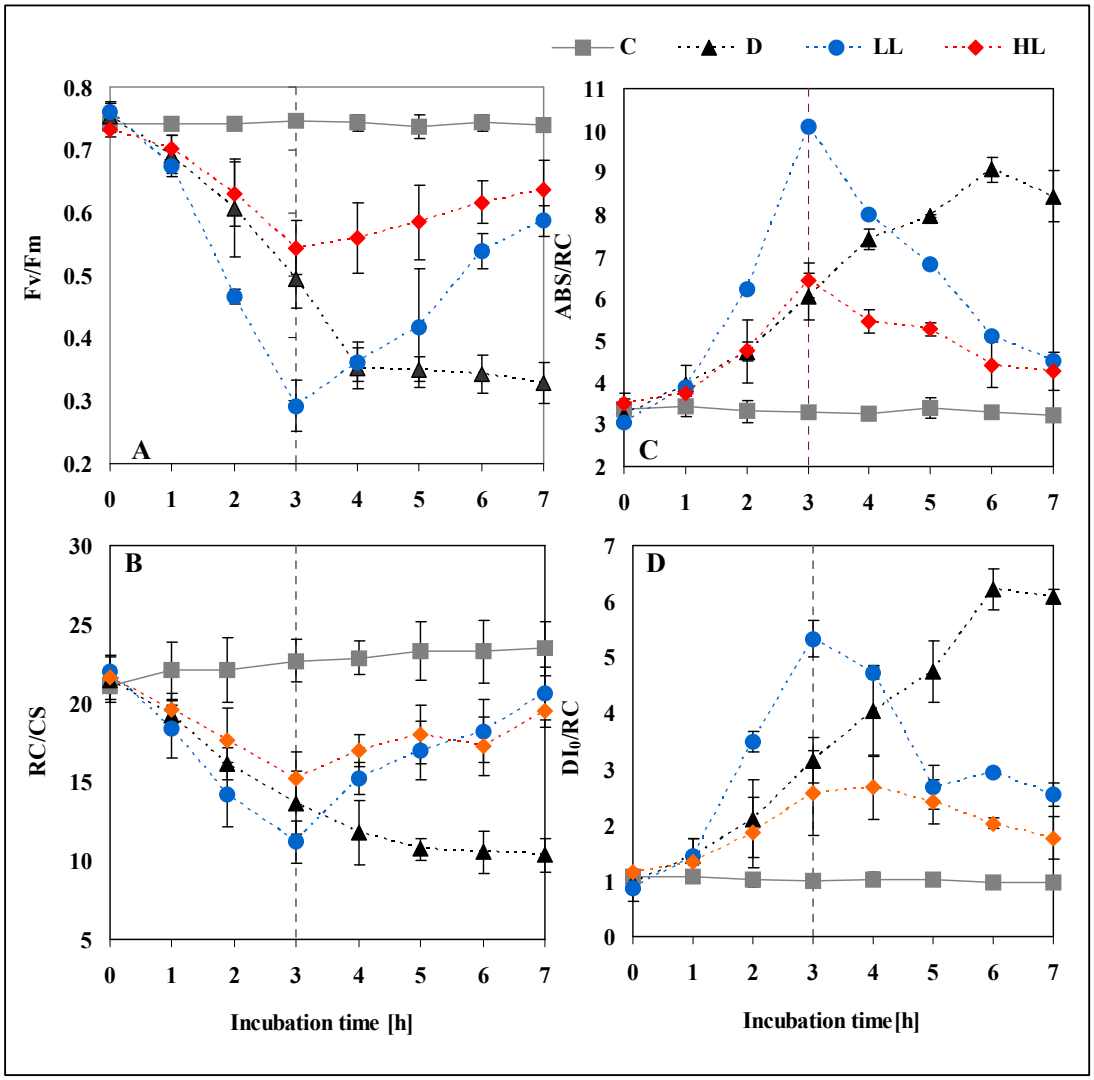


**FIGURE 5.** The kinetics of the (**A**) photosynthetic efficiency ($F_v/F_m$), (**B**) active reaction center density (RC/CS), (**C**) functional antenna size (ABS/RC) and (**D**) rate of dissipation energy per active reaction center ($DI_0/RC$), in cultures incubated in different conditions of illumination during irradiation with 0.42 mW $cm^{-2}$ UVB and recovery. The values represent the means ± SD of five independent experiments. For additional details, see legend Figure 3.

Consequent to increased antenna size, the surplus energy dissipation per number of active reaction centers ($DI_0/RC$) also increased, especially in LL+UVB

conditions (Fig. 5D). This rise in energy dissipation occurred in parallel to an increase in the inactivation of reaction centers, which probably were transformed into dissipative sinks for the excitation energy following the UVB treatment (Tevini et al., 1991). The density of active reaction center per cross section (RC/CS) was more significant affected in LL+UVB conditions (Fig. 5B). Recovery was fully accomplished in LL-UVB(R) and only partially in HL-UVB(R) cultures. In D-UVB(R) conditions, recovery did not occur, the density of reaction centers following a slightly continuous decrease.

Overall, the kinetics exhibited by these parameters indicated that the maximum quantum yield (Fv/Fm) changed as result of the inactivation of active reaction centers (RC/CS) and that the increase in functional antenna size (ABS/RC) in parallel to the decrease in RC/CS determined a similar increase in the dissipation rate of the excitation energy surplus ($DI_0$/RC) (Fig. 5A-D).

## 1.3. PHOTOCHEMICAL AND NON-PHOTOCHEMICAL QUENCHING CAPACITY OF UVB-IRRADIATED PHOTOSYNTHETIC APPARATUS

Light energy absorbed by Chl of photosynthetic organisms drives the photosynthesis and is also dissipated as heat and fluorescence. The energy distribution between photochemical activity and thermal dissipation can be estimated as photochemical and non-photochemical quenching. Different quenching mechanisms which increase the portion of dissipated energy and which are reflected as the qN parameter may be separated into several components with different relaxation kinetics: qE, high-energy quenching which is related to the formation of the pH gradient (quickly relaxing component, $t_{1/2}$<1 min); qT, quenching related to state transition, which changes the delivery of excitation energy by phosphorylation and migration of light-harvesting complex (LHCII) from PSII towards PSI (medium relaxing component, $t_{1/2}$>5±10 min), and qI, photoinhibitory quenching (slowly relaxing component, $t_{1/2}$> 30 min) related to photoinhibitory damage to PSII and requiring a repair mechanism, e.g. the synthesis and insertion of the reaction center protein $D_1$ of PSII (Jahns and Krause, 2000). Investigation of quenching properties of the photosynthetic apparatus, exposed to UVB, was made by PAM fluorometry using a protocol described in Material and Methods 8.1. Among the parameters investigated, the operational quantum yield of PSII ($\Phi_{SPSII}$) (Fig. 6A) is a measure of

the proportion of the light absorbed by PSII that is used in photochemistry. It is decreased about 92% in D+UVB and 63% in LL+UVB-cultures, whereas in HL+UVB condition it is reduced to 44%. Changes in operational quantum yield of PSII can be attributable to differences in the capacity for electron flux on the reducing side of PSII, i.e. in situations with limiting consumption of NADPH, but also to down-regulation of PSII, since $\Phi_{SPSII}$ is the product of the PSII efficiency factor ((Fm'-F)/(Fm'-Fo')) and the PSII maximum efficiency ((Fm'-Fo')/Fm')) which is affected by antenna quenching (Fracheboud and Leipner, 2003). Calculating the electron transport rate (ETR) it can be seen that it also highly decreased in LL+UVB and D+UVB conditions. These data indicate that both parameters ($\Phi_{SPSII}$ and ETR) are affected by the down-regulation of PSII by UVB radiation. In contrast, in HL+UVB culture, the ETR decreased with 31% of control, showing once more that HL protects against UVB stress (Fig. 6B). After the cessation of UVB treatment, both parameters recovered in the cultures incubated in light conditions, but they followed to decline in D. This is further evidence pointing toward PAR as an important factor contributing to protection for the photosynthetic apparatus and aiding its recovery from stress.

A decrease in the electron transport resulted in a decrease in the photochemical quenching capacity (qP) and, subsequently, to an increase in excitation pressure (described as 1-qP) exerted by UVB on the PSII. Photochemical quenching (qP) is an estimate of the open reaction centers (Maxwell and Johnson, 2000), being equivalent in value with the ratio $Q_{Aox}/(Q_{Aox}+Q_{Ared})$ (Ivanov et al., 2006). It can be seen that UVB induced a reduction in the $Q_A$ pool, this effect being more pronounced in D+UVB (83%) as in LL+UVB (47%) and HL+UVB (32%), respectively (Fig. 6C). The accumulation of reduced $Q_A$ molecules ($Q_A^-$) led to the increase in the excitation pressure exerted by UVB on PSII. The excitation pressure (1-qP) increased to 265% over the corresponding control in LL+UVB and 195% in HL+UVB. An interesting fact is that in D+UVB, the excitation pressure increased to 158%, as compared to control, but it became higher (170%) after the cessation of UVB treatment, when in LL and HL conditions it recovered to the control levels (Fig. 6E). The increase in the excitation pressure was accompanied by a decrease in non-photochemical quenching capacity (qN), especially in D+UVB culture, where the values obtained for qN were about 76% lower than those calculated for the corresponding control (Fig. 6D). It has to be pointed out that it is difficult to interpret

the data obtained for qN in stress conditions which affect the Fm values (Fracheboud and Leipner, 2003). The results obtained for qN in UVB-treated cultures are below the values obtained for their corresponding controls, due to the fact that UVB affected the Fm values more than the Fm´ ones. Nevertheless they are given to accentuate the differences of magnitude between the cultures responses to UVB which is influence by PAR. The dissipation of excess energy as heat can be estimated with higher accuracy from the values calculated for the $DI_0$/RC, which is increased in UVB-irradiated cultures as compared to the control cultures (Fig. 5D).

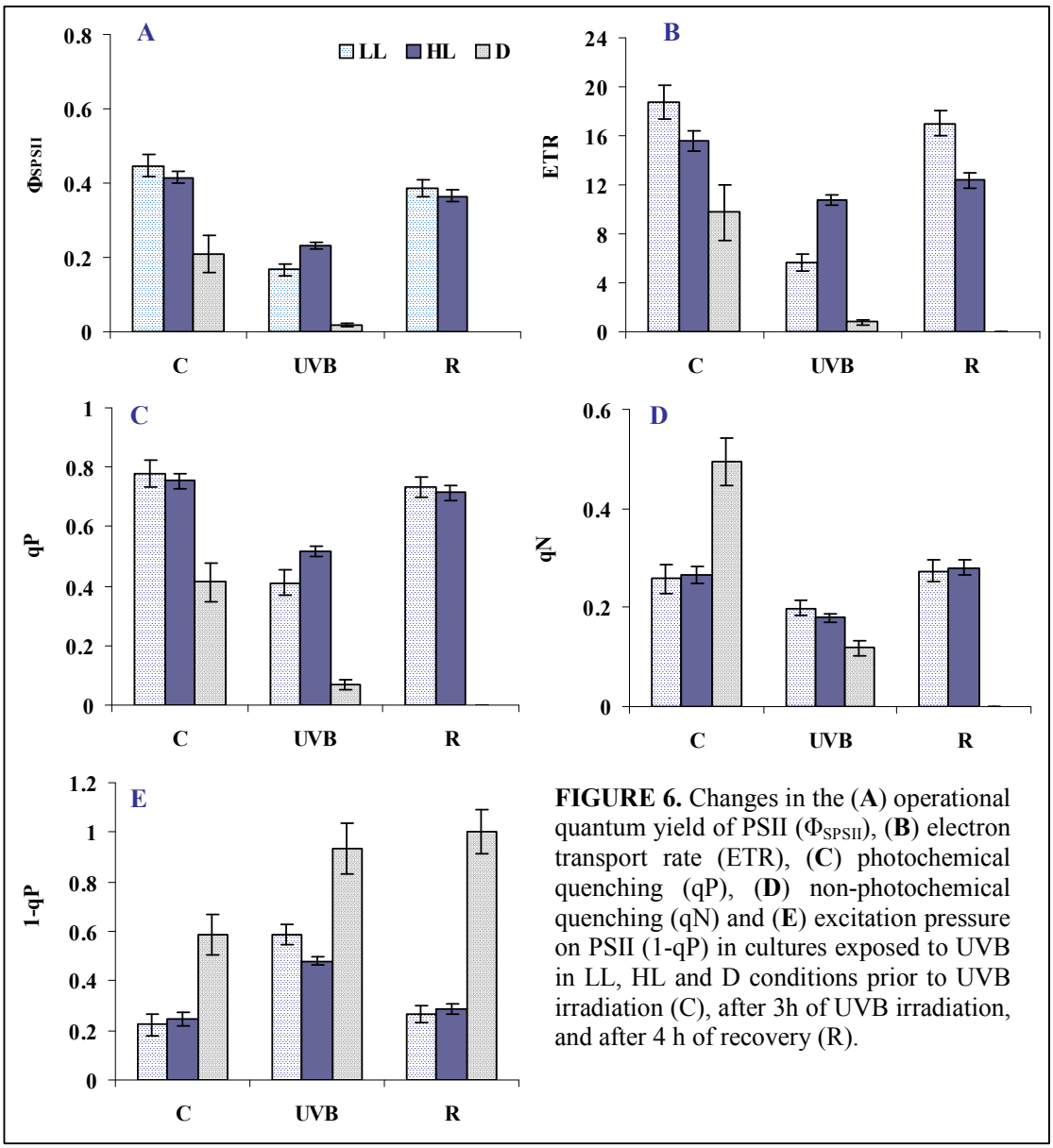


**FIGURE 6.** Changes in the (**A**) operational quantum yield of PSII ($\Phi_{SPSII}$), (**B**) electron transport rate (ETR), (**C**) photochemical quenching (qP), (**D**) non-photochemical quenching (qN) and (**E**) excitation pressure on PSII (1-qP) in cultures exposed to UVB in LL, HL and D conditions prior to UVB irradiation (C), after 3h of UVB irradiation, and after 4 h of recovery (R).

To give more insights into the dynamics of changes induced by UVB, a thorough analysis of fluorescence quenching was made step-by-step in cultures exposed to UVB for 11 h and additional recovery of 4 h (Fig. 7). The cell density of cultures was doubled, in order to attenuate the UVB stress effect. Analytically, the operational quantum yield of PSII ($\Phi_{SPSII}$) decreased as much as the maximum quantum yield (Fv/Fm), mainly after 5 h of UVB exposure. Most sensitive to UVB seems to be the rate of electron transport (ETR), which registered a more pronounced decrease from the 5$^{th}$ hour of UVB treatment. This reduction is simultaneous to a large increase in the excitation pressure exerted by UVB on PSII.

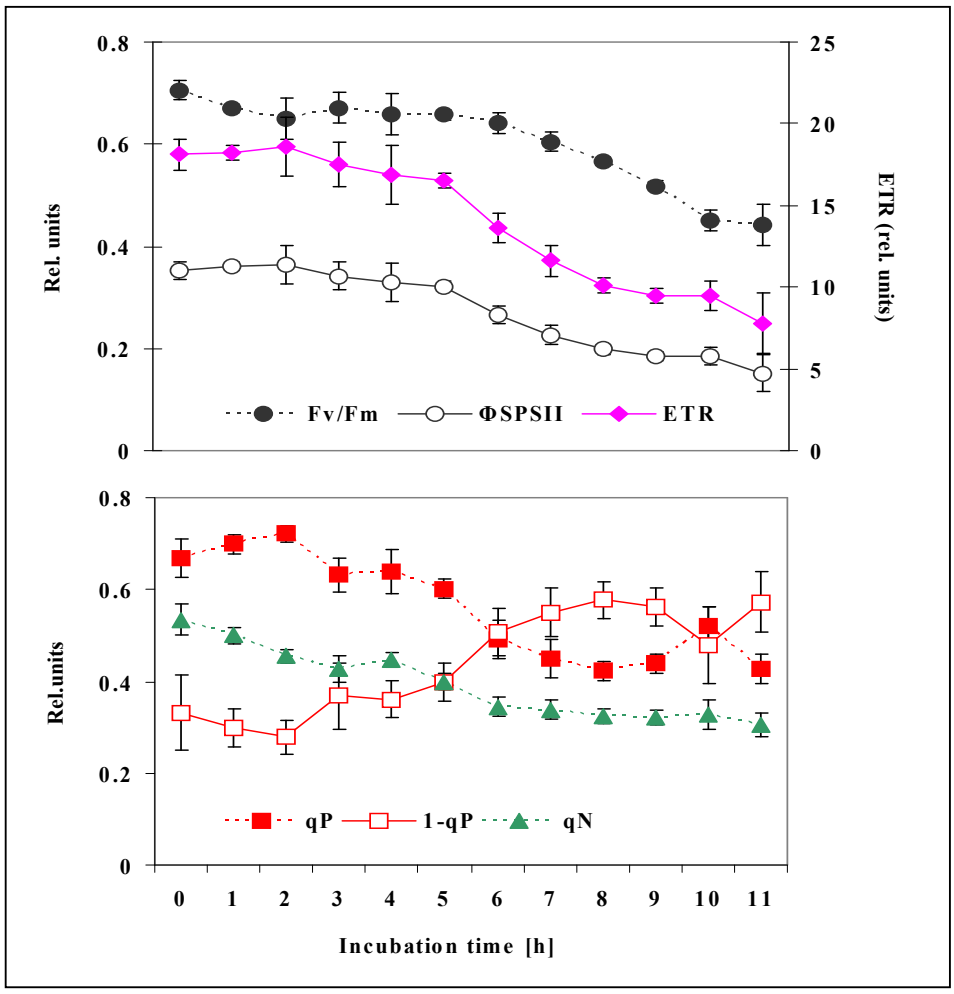


**FIGURE 7.** Changes in quenching kinetics over a period of 11 h exposure to UVB irradiation of cultures with increased PCV for the attenuation of UVB stress effect. The values given for each parameter are those measured at steady-state (after a period of 6 min exposure to 110 µmol $m^{-2}$ $s^{-1}$, with saturated pulses given at every 30 s). The parameters were calculated following the equations described in Material and Methods 8.1.

As long as the excitation pressure does not significantly change, the ETR exhibited a slow decline. From the 5$^{th}$ hour of exposure the excitation pressure increased rapidly and ETR registered a similar decline. The reduction in the ETR is followed by a decrease in quenching parameters (qP and qN) (Fig. 7).

## 2. CHANGES IN THE PSII HETEROGENEITY UPON UVB IRRADIATION

To assess the PSII heterogeneity, fluorescence transients were measured in samples treated or not with DCMU. This is an inhibitor of electron transfer from $Q_A$ to further electron acceptors, because DCMU binds instead of PQ molecule to the $Q_B$ pocket in the $D_1$ protein of PSII (Trebst, 1980; Strasser et al., 2000; Lazár et al., 2003).

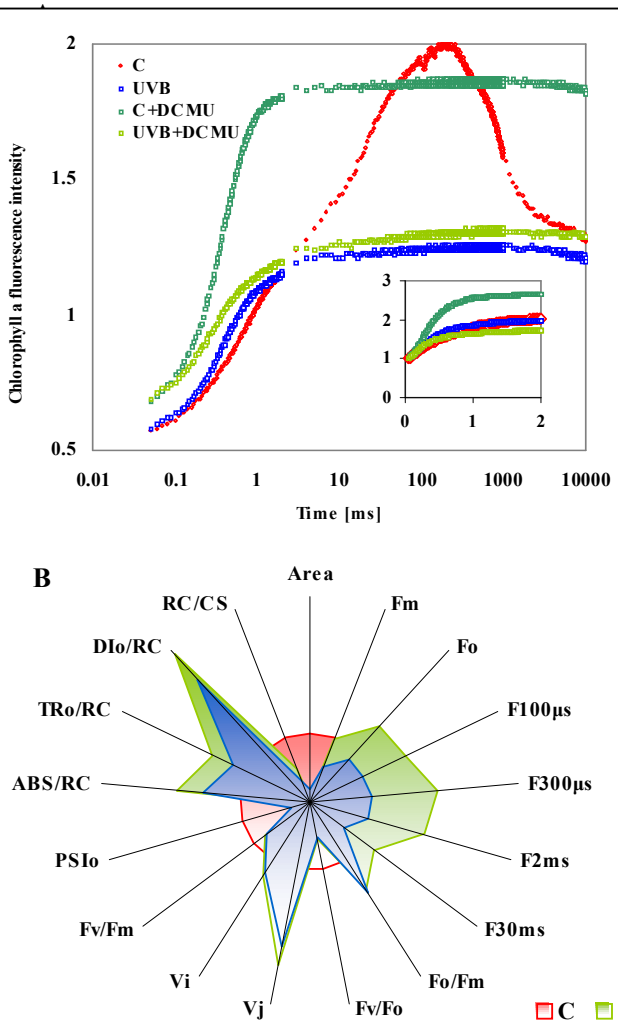


**FIGURE 8.** (**A**) O-J-I-P transients on a logarithmic scale in control and UVB-irradiated cultures prior and after incubation with 100 µM DCMU for 10 min in darkness. The inset shows the fluorescence of O-J phase on linear scale. (**B**) Radar plots of control+DCMU (*green circle*) and control+UVB (*blue circle*) as compared to control (*red circle*), which show the similarity of UVB and DCMU effects on the photosynthetic apparatus. This suggests that UVB radiation induces the blockage of electron transfer beyond $Q_B$ as DCMU does (*see* Table 2-Material and Methods for parameter definitions)

Due to the impossibility of electron transfer from $Q_A$ to the further electron acceptors when DCMU binds to the $Q_B$ pocket, the fluorescence signal during Chl fluorescence rise goes steeply up and reaches its maximal level at the position near the J step (Fig. 8A). The investigation of fluorescence transients in control samples treated with DCMU and control samples after UVB exposure revealed that there is a great similarity between the action mode of DCMU and that of UVB radiation (Fig. 8B). Both factors induced blockage of electron transport to $Q_B$. Concerning the effect of DCMU in UVB treated samples, it has to be noted that DCMU did not significantly change the UVB effects on the PSII photochemistry (Fig. 8A). Although it was postulated that UVB affects the $Q_B$ binding pocket in D1 protein (Vass et al., 1996) the measurements of oxygen evolution in the cells poisoned with DCMU after UVB irradiation (data not shown), suggested that the DCMU concentration was enough to inhibit the $O_2$ evolution to the same extent as that obtained for cells not treated with UVB.

The biphasic fluorescence rise in DCMU-poisoned samples (i.e. the J–I rise followed by the I–P rise; Fig. 8A) was suggested to reflect, in addition to an accumulation of different combinations of reduced $Q_A$ and $Q_B$ (Stirbet et al., 1995, 1998; Strasser and Stirbet, 2001) the PSII heterogeneity (Strasser et al., 1995; Lazár et al., 2003). The analysis of transients obtained with and without DCMU, helped in the investigation of PSII heterogeneity in UVB irradiated cultures. Two major fractions denoted as PSII-α and PSII-β reaction centers were distinguished with respect to the PSII antenna heterogeneity (Melis & Homann, 1975, 1976; Strasser, 1978; Lazár et al., 2001). This involves the PSII antenna size heterogeneity and the heterogeneity in energetic connectivity between PSIIs. PSII-α reaction centers are mainly localized in the grana region of the thylakoid membrane and are characterized by a large LHCII and the possibility of excited states transfer between PSII units. This transfer is reflected in a non-exponential (sigmoidal) fluorescence rise when measured with DCMU. On the other hand, PSII-β reaction centers are mainly localized in the stromal region of the thylakoid membrane and are characterized by about 2.5 times smaller LHCII when compared to LHCII of PSII-α and impossibility of the excited states transfer between PSIIs. On the basis of biochemical (Ort and Whitmarsh, 1990) and fluorescence (Melis, 1985; Lavergne and Leci, 1993) methods it was found that about 20 – 40% of PSII cannot reduce $Q_B$ and PQ pool. Thus, these PSII centers were called

$Q_B$ non-reducing PSII centers or inactive (in $Q_B$ reduction) PSII centers as different from the $Q_B$ reducing (or active in $Q_B$ reduction) ones that can reduce $Q_B$ and PQ pool. In mature leaves it was found that there is the same amount of the PSII-β reaction centers as the amount of the $Q_B$ non-reducing PSII centers (Melis, 1985; Ghirardi and Melis, 1988). This may not be true, especially for developing plant material where the amount of PSII-β significantly exceeds the amount of the $Q_B$ non-reducing PSII centers (Ghirardi and Melis, 1988). It was also suggested that the $Q_B$ non-reducing PSII centers can be of the α-type (Lavergne and Leci, 1993).

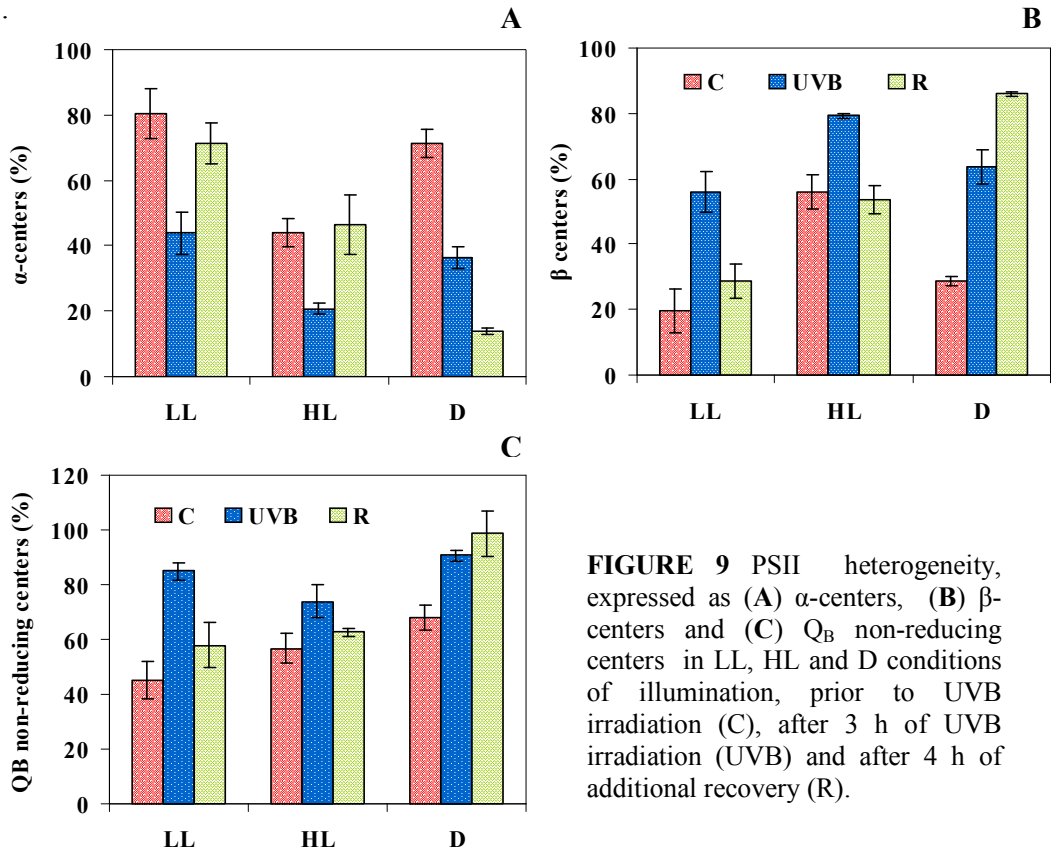


**FIGURE 9** PSII heterogeneity, expressed as (**A**) α-centers, (**B**) β-centers and (**C**) $Q_B$ non-reducing centers in LL, HL and D conditions of illumination, prior to UVB irradiation (C), after 3 h of UVB irradiation (UVB) and after 4 h of additional recovery (R).

## 2.1. PSII-α AND PSII-β CENTERS

With regard to the heterogeneity of PSII reaction centers, it is found that UVB preferentially inhibits the functionality of PSII-α concurrent with an increase in the percentage of $Q_B$ non-reducing centers (Figs. 9A, C). In LL+UVB condition, the amount of PSII-α decreased about 50 % of control value but recovered after the

cessation of UVB treatment. In opposition, HL+UVB also induced a 40% decrease of α-centers, similarly to D+UVB culture. In darkness, recovery was absent, which indicates that the damage induced by UVB on PSII-α centers functionality was irreversible (Fig. 9A). The decrease in the functionality of PSII-α centers was accompanied by an increase in the activity of PSII-β centers (Fig. 9B).

2.2. $Q_B$ NON-REDUCING CENTERS

The quantification of $Q_B$ non-reducing centres (Fig. 9C) shows that the inactivation is higher in LL (90 %) than in D (50 %) or HL (35 %). At recovery, the largest amount of $Q_B$ non-reducing centers becomes active again in the presence of light. In contrast, in absolute darkness the amount of $Q_B$ non-reducing centers continues to increase. Due to the fact that the kinetics of $Q_B$ non-reducing centers are similar to those of PSII-α reaction centers, one can assume that the highest proportion of $Q_B$ non-reducing centers resulted from the UVB-induced inactivation of PSII-α reaction centers. This effect is strongly expressed in LL+UVB and D+UVB conditions, but it is reversed after the cessation of UVB treatment only in light, showing once more that the recovery of PSII functionality is a light-dependent process (Fig. 9C).

## 3. MAXIMAL PHOTOSYNTHETIC RATE (OXYGEN EVOLUTION) IN UVB-IRRADIATED CULTURES

Polarographic measurements of oxygen evolution prior, after UVB exposure and after recovery indicate that UVB radiation induced a decrease in the maximal net photosynthetic rate (Fig. 10). Compared to the corresponding control cultures, the maximal net photosynthetic rate values obtained for LL+UVB and HL+UVB cultures were reduced by 54% and 27%, respectively, while in D+UVB the decrease induced by treatment was about 80%. The decline induced by UVB in the maximal net photosynthetic rate under LL-UVB(R) and HL-UVB(R) conditions was rapidly recovered to a significant level (between 80% and 95% for all treatments), while in D-UVB(R) condition no recovery was found (Fig. 10).

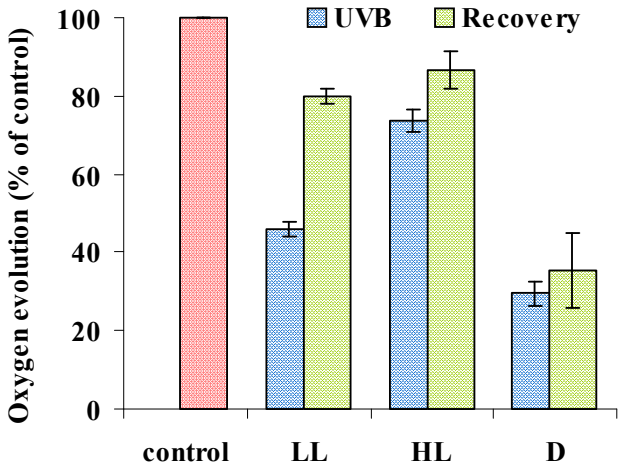


**FIGURE 10.** Maximal net photosynthetic rates (oxygen evolution) in cultures exposed to UVB and after recovery in LL, HL and D conditions as compared to the corresponding controls. The cell suspension density was adjusted to 10µl PCV/ml and the maximal photosynthetic rate was expressed in µmol $O_2$ mg $Chl^{-1}$ $min^{-1}$. The values on Y-axis are given in % of the corresponding control values.

## 4. STRUCTURAL CHANGES IN THE PHOTOSYNTHETIC APPARATUS AS RESPONSE TO UVB RADIATION TREATMENT

Biochemical analysis performed in cultures exposed to UVB showed that upon UVB treatment significant alterations occurred in the pattern of pigments (Chls and Car), intracellular polyamines and polyamines associated to thylakoids, as well as, in the structure of the chlorophyll-protein sub-complexes of light harvesting antenna associated to PSII.

### 4.1. QUANTITATIVE AND QUALITATIVE CHANGES IN THE PIGMENT POOL UPON UVB RADIATION

#### 4.1.1. Chlorophylls

The determination of Chl suggested that the cultures used as control generally show a state of adaptation that correlates with the light intensity applied during culturing. LL-exposed cultures possessed higher content of Chl comparative to HL-adapted cultures (Fig. 11A). On the contrary, cultures exposed to darkness contained less Chl compared to those incubated under light conditions. After UVB exposure, cultures exhibited higher Chl level than the respective control. No significant change in Chl level was recorded during the recovery period.

The values calculated for Chl a/b ratios were lower in light-incubated cultures than in darkness ones (Fig. 11B), showing clearly that cultures used as control were adapted to PAR intensity conditions. Chl a/b ratio is a factor indicating the LHCII

antenna size. Since Chl b is exclusively bound to LHCII antenna, a decrease in this ratio indicates a higher Chl b content and, consequently, a bigger LHCII (Anderson et al., 1988). As it was expected, the Chl a/b ratio was lowered in cultures exposed to UVB irradiation; this confirms that the antenna size increased during irradiation. During recovery, the Chl a/b ratio decreased in D-UVB(R) condition, whereas it increased to control level in LL-UVB(R) and HL-UVB(R) cultures (Fig. 11B).

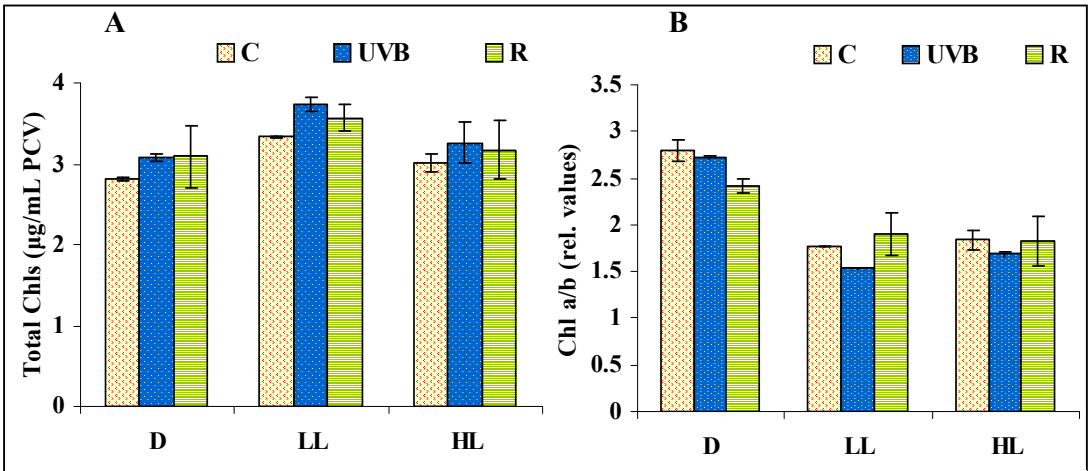


**FIGURE 11.** Total Chl amount (**A**) and Chl a/b ratios (**B**) in cultures incubated in LL, HL and D conditions, before (C), after 3 h of UVB radiation (UVB) and after additional 4h of recovery (R).

The fluctuations of Chl a/b ratios in UVB-irradiated *Scenedesmus* cultures, although not statistically significant are important because they correlated to the oscillations in antenna size. The changes that occurred in Chl content, as well as in Chl a/b ratio indicate that cultures show a highly adaptive behavior to the conditions applied during the experiments.

### 4.1.2. Carotenoids

Car (carotenes and xanthophylls) play different structural and functional roles in the photosynthetic antenna complexes of higher plants and algae. They can function as: (i) accessory light-harvesting pigments, (ii) structural entities within the LHCII, and (iii) molecules required in photoprotection of photosynthetic organisms from the potentially damaging effects of light (Demmig-Adams, 1990). The estimation of carotenes and xanthophylls levels by HPLC showed quantitative oscillations during UVB irradiation, as compared to the corresponding controls. Specifically, the

irradiation with UVB leads to increase in the amount of α- and β-carotene (car) level, whereas the neoxanthin (Nx) level is decreased. Also, UVB stimulates the increase in lutein (L) in detriment of loroxanthin (Lx), giving higher values of L/Lx ratios in both HL and LL conditions (data not shown). There is also a stimulation of vioaxanthin (Vx) biosynthesis, especially in HL+UVB culture (Fig. 12).

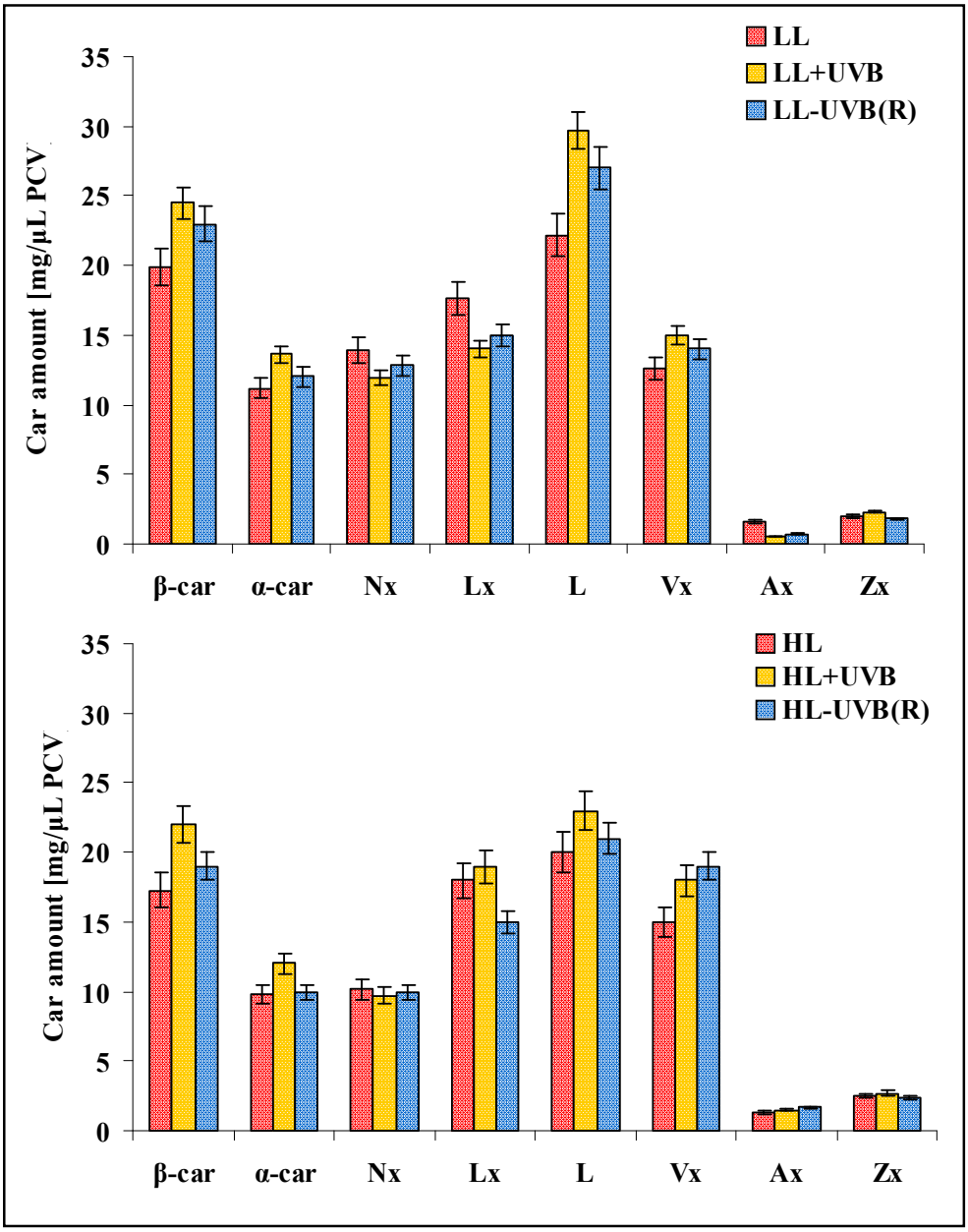


**FIGURE 12.** Carotenoid pattern investigated by HPLC in cultures exposed to UVB in LL and HL conditions, prior to UVB exposure (LL or HL), after 3 h of UVB irradiation (LL+UVB; HL+UVB) and after additional 4h of recovery (LL-UVB(R); HL-UVB(R)).

As depicted in Fig.13, the xanthophyll pool (Vx+Ax+Zx) increased during UVB irradiation in both LL+UVB and HL+UVB cultures but the ratio Vx/(Zx+Ax), which is an indicator of Vx conversion did not decrease upon UVB irradiation. The violaxanthin cycle, which is a well known mechanism of photoprotection contributing to the dissipation of excess excitation energy by high-energy non-photochemical quenching (qE), did not operate in cultures exposed to UVB. This confirms the data obtained by Pfündel et al. (1992) that UVB radiation inhibits the de-epoxidation of Vx (Vx cycle), which is responsible for the conversion of Vx into Zx.

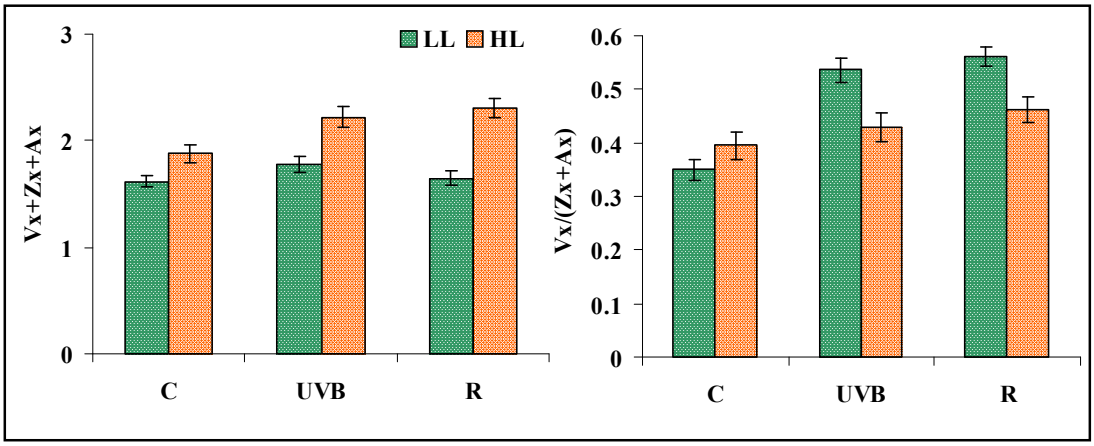


**FIGURE 13.** The xanthophyll pool (Vx+Zx+Ax) and the xanthophyll cycle (the conversion of Vx into Zx and Ax, here expressed as Vx/(Zx+Ax) in cultures incubated in LL and HL conditions, prior to UVB exposure (C), after 3 h of UVB exposure (UVB) and after 4 h of additional recovery (R).

An additional experiment performed in the presence of DTT, which is known to block the activity of Vx-deepoxidase (Pfündel et al., 1992) indicates that the PSII photochemistry decreased upon UVB irradiation independently of DTT addition (data not shown). This means that the increase in the dissipation of excess energy as heat is not related to the activation of the Vx cycle, considered an important non-photochemical quenching mechanism.

## 4.2. UVB INDUCED CHANGES IN THE STRUCTURAL ORGANIZATION OF THE LHCII

The photosynthetic sub-complexes and mainly the monomeric and oligomeric forms of LHCII were separated from the isolated thylakoid membranes prior to UVB irradiation (control), after 3 h of UVB irradiation and after an additional 4 h period

without UVB. Quantitative analysis of the isolated LHCII sub-complexes revealed that the UVB treatment caused an increase in the oligomeric fraction concurrently with a decrease in the monomeric one (Fig. 14).

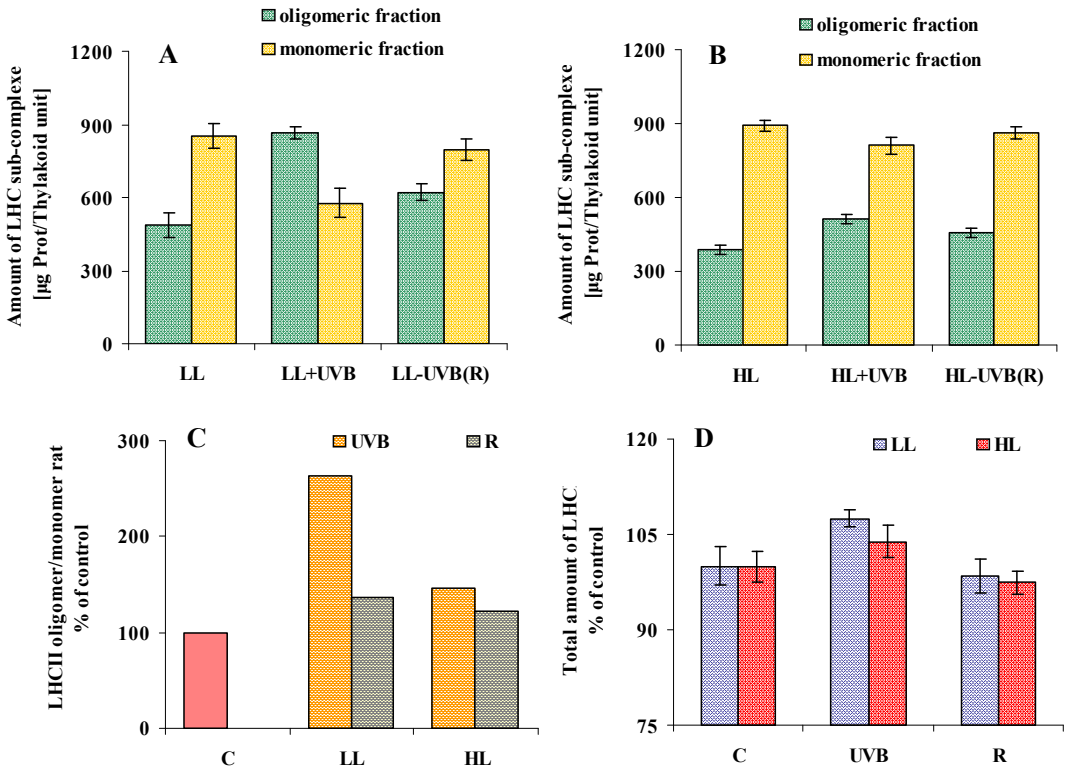


**FIGURE 14.** Changes in the oligomerization status of LHCII (**A-C**) and in the total LHCII protein amount (**D**) in cultures exposed to UVB in LL and HL conditions, at three experimental steps: prior to UVB irradiation (C), after 3h of UVB irradiation (UVB) and after additional 4h of recovery (R).

Comparative to the corresponding control values, the UVB-induced increase in the oligomeric fraction was higher in LL+UVB (77%) than in HL+UVB culture (33%) (Fig. 14A-B). The increase in the oligomeric fractions of LHCII combined with the decrease in the monomeric ones resulted in the increase of the oligomeric/monomeric fraction ratio during UVB exposure (Fig. 14C). In the LL +UVB condition, this ratio increased by about 163% over the corresponding control value, while in the HL+UVB condition this ratio increased by about 46%. Four hours after the cessation of UVB treatment, the oligomeric forms of LHCII decreased concurrently to the increase in the corresponding monomeric ones (Fig. 14A-C) and

the oligomeric/monomeric fraction ratio partially recovered. Although the oligomerization state of LHCII increases with UVB radiation, the total amount of LHCII proteins is slightly increased by the treatment, only in LL+UVB condition (Fig. 14D).

## 4.3 CHANGES IN THE AMOUNT AND PATTERN OF INTRACELLULAR AND THYLAKOID-ASSOCIATED POLYAMINES

The participation of polyamines in the assembly of the photosynthetic apparatus (reviewed in Kotzabasis, 1996) and their involvement in the photosynthetic activity (Kotzabasis and Senger, 1994) and chloroplast photodevelopment (Andreadakis and Kotzabasis, 1996), as well as in the regulation of LHCII size (Kotzabasis et al., 1999), prompted us to examine the changes that occur in the amount and pattern of intracellular and thylakoid associated-polyamines in cultures during UVB exposure and recovery periods. Data resulting from the quantitative determination of Put and Spm content have been used for the calculation of Put/Spm ratio, which may be considered an indicator of photoadaptation degree that cultures adopt in the experimental conditions used. As depicted in Figure 15, the total polyamine content increased in LL+UVB condition much more than in HL+UVB cultures. This response seems to be UVB specific and light regulated.

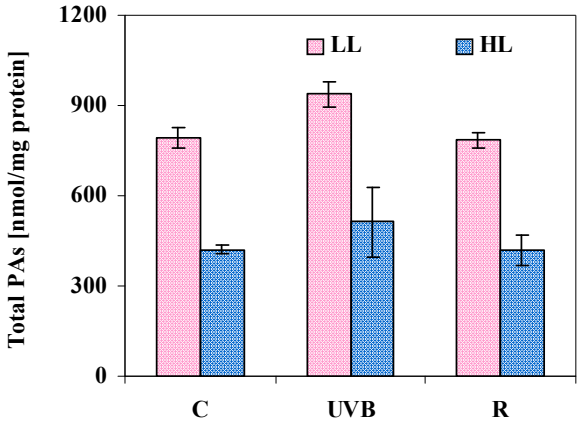


**FIGURE 15.** Intracellular polyamine content in LL and HL-cultures prior to UVB treatment (C), after 3h of UVB irradiation (UVB) and after 4 h of recovery (R) as determined by HPLC.

This increase in the content of total polyamines is due to the large accumulation of Spd to the detriment of Put (Fig. 16). A different pattern of changes

was found at the thylakoid level. The thylakoid membranes were isolated from cultures prior to UVB irradiation, after 3 h of UVB irradiation and after an additional 4 h period of incubation in the same PAR conditions without UVB.

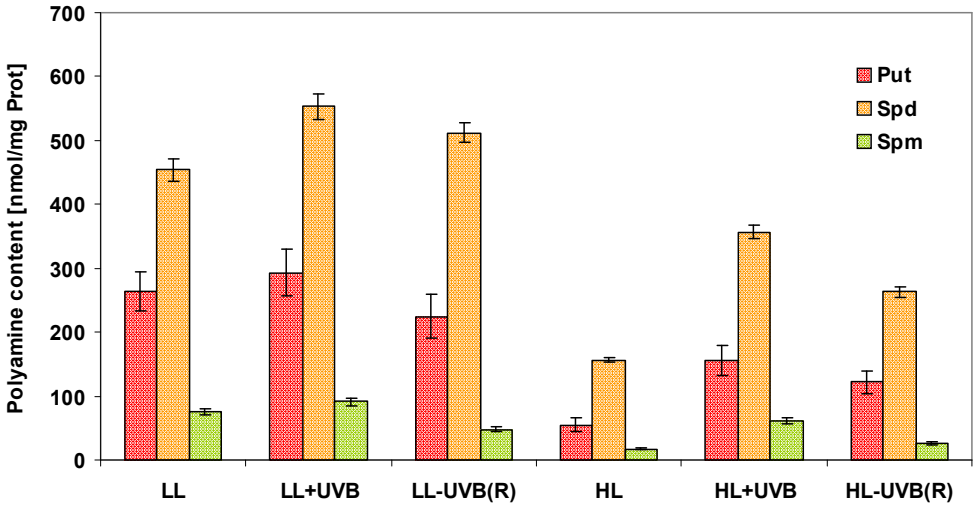


**FIGURE 16.** The pattern of cellular polyamines prior to UVB irradiation and after 3h of UVB irradiation in LL and HL cultures.

As illustrated in Fig. 17, the Put content in thylakoids decreased during UVB irradiation in LL+UVB culture, while in HL+UVB condition it was stable maintained. In contrast, Spd decreased in both experimental conditions with UVB exposure, whereas the amount of Spm-associated to thylakoids increased in LL+UVB and HL+UVB cultures, mainly in LL. These changes in the polyamine content led to an increase in the total polyamine amount bound to thylakoid membranes, especially in LL+UVB condition (Fig. 18A), as it was previously shown for the intracellular polyamines (Fig. 16). This again validates the observation that UVB induces an increase in the polyamine content, and the magnitude of this response is modulated by visible light.

The Put/Spm ratio in thylakoids also exhibited changes by UVB treatment. Considering the Put/Spm ratio as indicative of the adaptative state of the photosynthetic apparatus, it can be observed that UVB induced a reduction of this ratio, also in LL as well as in HL conditions. In agreement with Kotzabasis et al. (1999), a decrease in the Put/Spm ratio is an indicator of a LL-adapted photosynthetic apparatus, i.e. with a larger antenna size.

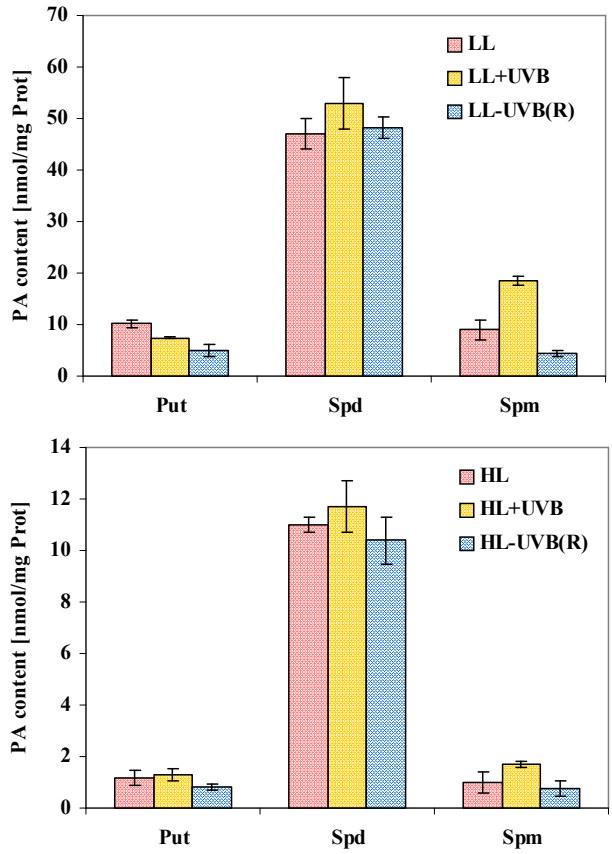


**FIGURE 17.** The pattern of thylakoid-associated polyamines in the thylakoid membranes isolated from LL and HL cultures prior to UVB irradiation and after 3h of UVB irradiation.

Indeed, the changes occurring in the size of antenna follow the same trend as those of the Put/Spm ratio measured in thylakoid membranes. In comparison to the corresponding control values, the lowest Put/Spm ratios was obtained in the LL+UVB culture that presented a larger increase in antenna size than the culture incubated in HL+UVB condition (Fig. 18B). After the cessation of UVB irradiation treatment, the Put/Spm ratios recovered in both LL and HL cultures. Assuming that changes in the Put/Spm ratio play a regulatory role of the antenna size (Kotzabasis et al., 1999), the data obtained from the investigation of polyamine pattern in thylakoids and whole cells suggest the involvement of thylakoid-associated Put and Spm in the regulation of LHCII antenna size, since there is a strong correlation between the changes occurring in the size of antenna and in the pattern of Put and Spm, during UVB irradiation and at recovery.

The antenna size is not mediated through changes in the cellular polyamines, which exhibited different kinetics than those obtained for the antenna size. Therefore, this is more evidence of the contribution of thylakoid-associated polyamines in the

regulation of LHCII size during UVB irradiation and supports the previous finding that the Put/Spm ratio in thylakoids regulates the antenna size (Kotzabasis et al., 1999).

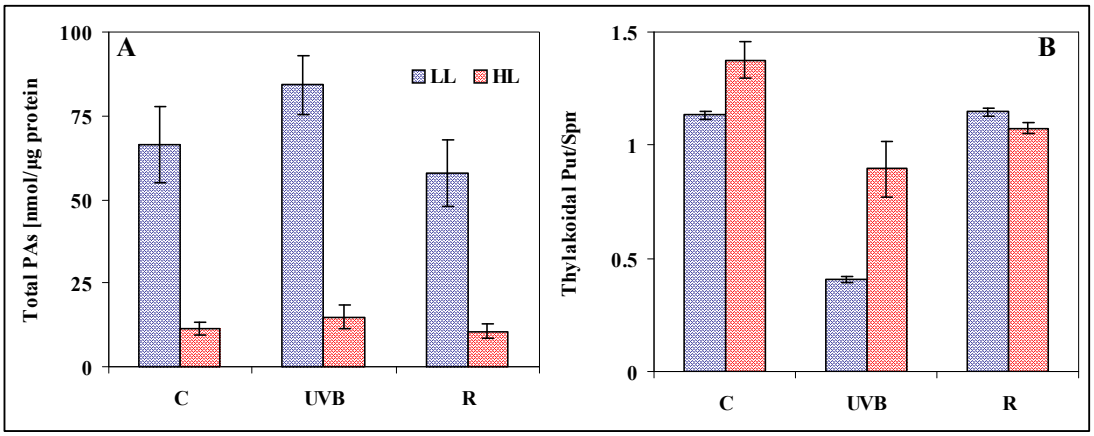


**FIGURE 18. (A)** Total content of thylakoid-associated polyamines and **(B)** the Put/Spm ratio in membranes isolated from cultures irradiated with UVB in LL and HL conditions at three experimental steps: prior to UVB radiation (C), after 3 h of UVB radiation (UVB) and after 4 h of recovery (R).

## *DISCUSSION*

The main target of UVB radiation in the photosynthetic apparatus is the PSII, but the primary sites responsible for the suppression of the PSII activity by UVB is still a matter of debate (Tevini, 2004). The different results can be explained by different UVB radiation regimes, which were sometimes very unnatural. Several different target sites have been proposed (Bornman, 1991). These include the reaction center of PSII, the light harvesting complex (LHCII), and the acceptor/donor side of PSII.

The data presented herein show that both the LHCII and reaction center of PSII can be affected by UVB radiation, but the intensity of damage depends on the irradiation conditions applied during treatments. In the context of the enhancing damaging effects of UVB on photosynthesis, as predicted by different ozone depletion scenarios, the results obtained from the experiments performed on *Scenedesmus obliquus* suggest that there is a fine mechanism that regulates the photosynthetic behavior to UVB radiation assuring the maximal efficiency with the minimal losses. The mechanism that adjusts the molecular structure, conformation and functioning of the photosynthetic apparatus to UVB is acting through LHCII antenna regulation and probably is represented by polyamines.

1. The sensitivity to UVB radiation results from changes in the molecular structure, conformation and function of the photosynthetic apparatus

The time/dose response of the photosynthetic apparatus to UVB indicates that the UVB damage is the result of cumulative fluences of the changes induced in PSII photochemistry (Fig. 2). These results are in agreement with previous experimental data, which showed that the maximum quantum yield of PSII (Fv/Fm) is down-regulated by UVB radiation through photochemical damage of PSII (Tevini and Teramura, 1989; Strid et al., 1990; He et al., 1993; Teramura and Sullivan, 1994; Day and Vogelmann, 1995; Krause et al., 1999; Rajagopal et al., 2000; Jansen et al., 2003). However, primary photochemistry of PSII (the charge separation, recombination and stabilization), non-radiative loss of excited states in light harvesting antennae and excited states quenching by oxidized plastoquinone (PQ) molecules from the PQ pool seem to be the main factors controlling the maximum quantum yield of PSII photochemistry as expressed by the Fv/Fm ratio (Lazar, 2003). In addition, protein degradation driven by UVB radiation was

found to attend this loss in PSII reaction centers functionality (Friso et al., 1994). Indeed, the data obtained here show that there is a linear correlation between the decrease in PSII photochemistry and the inactivation of reaction centers (Figs. 2C, 5A, 5B)

Considering the high degree of similarity that exists between UVB and DCMU action (Fig. 8B), the quenching effect of UVB radiation seems to result from the blockage of electron transport to $Q_A$ (Figs. 6-7). It is well-known that the PQ pool in dark-adapted photosynthetic systems usually remains in the predominantly oxidized state (Lázar, 2003). Upon UVB irradiation, the fast rise of Chl fluorescence from $F_0$ to Fm implies that the $Q_A$ is in a predominately reduced state (Fig. 3). Since $Q_A$ is in quasi-equilibrium with $Q_B$ and the PQ pool these results imply that the PQ pool should be also in a partially reduced state in dark-adapted samples. These observations are in agreement with previous reports indicating that the UVB radiation and DCMU cause the same type of inhibition of the re-oxidation of the primary electron acceptor $Q_A$ of PSII and confirms the general understanding that the primary site of the inhibition of electron transport is at the site of PQ (Masi and Melis, 1997).

From earlier observations that the variable fluorescence is lost in concert with the D1 protein, one can assume that the $Q_B$ sites are lost in parallel to D1 protein and photochemical efficiency (Figs. 4, 5A). Remaining $Q_B$ sites are supposed to be connected to $Q_A$ and the efficiency of such connections depends on the redox state of the intersystem electro chain, i. e. largely-reduced PQ pool in dark adapted samples. An alternative explanation for the fast rise could be the blockage of the electron transport between $Q_A$ and $Q_B$ for other reasons than a reduced PQ pool which keeps the $Q_B$ reduced and unable to accept electrons from $Q_A$. The investigation of ETR also indicates that UVB induced a decrease in the rate of electron flow on PSII (Figs. 6B, 7).

The accumulation of reduced quinones represents the first order cause of the increased excitation pressure on PSII (Figs. 6D, 7). An increased excitation pressure interferes with a reduction of the reaction center functionality (Figs. 5B, 9C). This ultimately, means the reduction in the photosynthetic apparatus capacity for the photochemical use of the absorbed energy, in the so-called process of photochemical quenching (Figs. 6C, 7). The qP parameter, describes not only the capacity for the photochemical quenching of the excitation energy, but also it is a measure for the estimation of $Q_A$ reduced accumulation. Decreasing the qP (Figs. 6C, 7), the excitation pressure on PSII increases (Figs. 6D, 7) and the reaction centers are inactivated (Fig.

5B). Due to all these changes, the photosynthetic apparatus reacts by increasing the size of antenna (Figs. 5C, 14). This is the solution chosen to assure the dissipation of surplus energy (Fig. 5D) and to maintain some equilibrium between energy absorption and usage. Two mechanisms contributed to the increase in the antenna size:

a) the oligomerization of LHCII (Fig. 14) and

b) the transformation of the $Q_B$ non-reducing centers (Figs. 5B, 9C) in dissipative sinks for the excitation energy (Strasser, 1978).

Both mechanisms are simultaneously operating in the photosynthetic apparatus during UVB exposure. The increase in antenna size and rate of energy dissipation was prolonged in proportion to UVB radiation (Figs. 5C-D). Whether the inactivated reaction centers contribute to the dissipation of energy surplus, the increase of LHCII is somehow curious (Fig. 14A-C). It is well known that in nature, plants increase their LHCII size when the amount of light energy available for the photosynthetic processes is reduced. A major environmental variable as UVB radiation can perturb the equilibrium between energy input and energy consumption and induce photosynthetic alterations. Because of this, exposure to UVB may result in the photosynthetic apparatus absorbing more light than can be readily dissipated through carbon fixation. In natural conditions, there are several possible mechanisms to maintain the energy balance: (a) alterations in light harvesting and primary photochemistry to decrease the amount of light energy absorbed; (b) increased rates of cyclic or pseudocyclic electron transport (Mehler reaction); (c) increased rates of photorespiration or chlororespiration; and (d) increased enzymic activity of the Calvin cycle, resulting in higher $CO_2$ fixation rates (Huner et al., 1998).

A possible explanation for the observed increase in the size of LHCII (Fig. 14C) may be that the photosynthetic apparatus tries to compensate for functional losses induced by the inactivation of reaction centers by enhancing the photosynthesis. Due to the limitation of ETR (Figs. 6B, 7), which could occur by PSII damage (Vass et al., 1996), the energy absorbed can not be efficiently used in photosynthesis (Figs. 6C, 7), so that the bigger antenna now plays its second major role, to dissipate the excess energy. There is a strong correlation between the oligomeric/monomeric fraction ratio of LHCII (Fig. 14C), ABS/RC (which expresses the size of antenna) (Fig. 5C) and $DI_0$/RC (the rate of excess energy dissipation) increasing (Fig. 5D). This can be explained by previous findings showing that an increase in the oligomerization status of LHCII leads

to aggregation (Ruban et al, 2005), a phenomenon which assures a high rate of energy dissipation.

The data obtained from the measurements of non-photochemical quenching in UVB radiated culture are somehow in contradiction with those provided by the JIP-test. Although the $DI_0$/RC increased (Fig. 5D), the qN decreased (Figs. 6E, 7). The explanation consists in that the UVB inactivated reaction centers (Fig. 5B) represent an obstacle in the accurate determination of Fm value. This was also demonstrated by previous investigations of UVB effects on the photosynthetic apparatus, which suggests that the increase in Fs is a result of UVB induced quenching on Fm and that Fm is more susceptible to UVB as Fm´ (White and Jahnke, 2002). The increased excitation pressure associated with the decline in the photochemical quenching capacity of the photosynthetic apparatus resulted in the reduction of both quantum yield efficiencies in dark- (Fv/Fm) and light-adapted ($\Phi_{SPSII}$) cultures (Figs. 5A, 7) and maximal net photosynthetic rate (Fig. 10).

An interesting aspect of the photosynthetic apparatus behavior to UVB is represented by the changes induced in the PSII heterogeneity. It is postulated that PSII comprised two populations of RCs, namely PSII-α and PSII-β centers. They differ in antenna size and localization in the thylakoid membranes. In addition to the difference in the antenna size heterogeneity, there is a functional difference regarding the energetic connectivity. As depicted in Fig. 9A-B, UVB preferentially inactivates the PSII-α centers, while the activity of PSII-β centers is enhanced. This response suggests that an enlargement of antenna is associated with the loss in the functionality of reaction centers possessing a bigger antenna as compared to those having a smaller antenna. In this context, the UVB-induced oligomerization of LHCII (Fig. 14C) has the role to contribute to the dissipation of the surplus of the excitation energy as heat (Fig. 5D). The quantification of $Q_B$ non-reducing centers represents another line of evidence supporting the inactivation of reaction centers upon UVB irradiation (Fig. 9C). Assuming that $Q_B$ non-reducing centers originate from those with a larger antenna (since they are inactivated first) the percentage obtained for them is higher than the corresponding inactivation of PSII-α centers (Fig. 9A). It can be proposed that a part of PSII-β centers (Fig. 9B) are also inactivated and this may occur in the later phase of damage when the functionality of PSII, as revealed by the quenching analysis (Fig. 7), suddenly declined after 5 h of UVB treatment.

The increase in the oligomerization status of LHCII is accompanied by changes in the amount and pattern of polyamines, as investigated at both the whole cell and thylakoid membrane levels. UVB induced an increase in the intracellular accumulation of Spd (in both LL and HL-incubated cultures), whilst it decreased Put (Fig. 16). In the thylakoid membranes, there is an increase in the accumulation of Spm parallel to the decrease in Put and Spd content (Fig. 17). These changes at thylakoid level resulted in the diminution of Put/Spm ratio upon UVB radiation (Fig. 18B) and this is in agreement with the finding of Kotzabasis et al. (1999) that a photosynthetic apparatus with a bigger antenna possess a lower Put/Spm ratio than one having a smaller antenna. Thus, Put/Spm ratio may be considered an indicator of the antenna size not only during photoadaptation, as previously showed by Kotzabasis et al (1999), but also in stress conditions, as it happens when the photosynthetic apparatus is exposed to UVB.

Concurrent to the changes occurring in the pattern of LHCII and polyamines, UVB induced an increase in the accumulation of β-carotene and lutein (Fig. 12). β-carotene seems to act as an efficient scavenger of active oxygen species, since the methods used for the estimation of potential oxidative stress induced by UVB showed no important signs of AOS accumulation (*see* Results and Discussion-*Chapter III*). This is in agreement with the observation that in *Dunaliella* the accumulation of β-carotene prevents the UV-induced photosynthetic damage through absorbtion of UVB and BL (White and Jahnke, 2002). Lutein is important for the stabilization of LHCII sub-complexes (Bishop, 1996) and its increase is needed to achieve a bigger antenna with a larger capacity for energy dissipation (high-quenched antenna status) (Fig. 12). Similarly, the Chl a/b ratio also shows lower values upon the UVB irradiation, demonstrating once more the increasing antenna size (Fig. 11B).

Apart from the damaging effect of UVB radiation on PSII functionality, the basic behavior adopted by the photosynthetic apparatus upon UVB exposure mimics LL-photoadaptation responses. A similar conclusion was drawn by Fagerberg and Bornman (2005), which demonstrated that the alterations induced by UVB in leaf anatomy and chloroplast structure in *Brassica* were similar to the changes induced by transition from HL to LL, although there was no significant difference in chlorophyll a, b or carotenoid content compared to control plants.

2. Light signature on the photosynthetic apparatus behavior adopted upon UVB exposure

Fluorescence induction measurements indicate clearly the increased sensitivity of the photosynthetic apparatus when it is exposed to UVB in D and LL conditions (Fig. 3). From the analyses of transients exhibited prior and after UVB exposure, one can easily see that the quenching of $F_m$ is higher in LL and D as in HL being accompanied by an increase in the ground-state fluorescence ($F_0$). Moreover, the shapes of transients are highly affected by D or LL intensity culminating with the disappearance of I-step upon UVB irradiation (Fig. 3). This finding is in agreement with previous results showing that besides Fm reduction, UVB induced an increase in $F_0$ in LL conditions (Heraud and Beardall, 2000), whilst in HL condition of illumination $F_0$ is not changed (Krause et al., 1999). Since $F_0$ originates from the antenna-associated Chl a, its increase is indicative of decreased energy transfer from LHCII to PSII cores and/or less efficient energy processing in the cores (Lazár, 2003).The following mechanistic explanations for the increase in $F_0$ have been proposed:

(1) the formation of a PSII RC with the quinone acceptor stabilized in the protonated reduced form ($Q_AH$) or

(2) the (partial) disconnection of PSII RC from the antenna complexes (Lavergne and Joliot, 1996).

The data accumulated until now supports that upon UVB radiation, the increase of $F_0$ suggests a blockage in electron flow out of PSII (Heraud and Berdall, 2000), which indicates the first mechanism as operating during UVB radiation (Fig. 7). In agreement with this finding, the results described here show that LL induced a larger reduction of $Q_A$ pool as in HL (Figs. 6C, 7) due to the higher inhibition of ETR, as estimated from the fluorescence quenching investigations (Figs. 6B, 7). This caused the inhibition of RC functionality, which is maximal in LL conditions (Figs. 5, 9C). The inactivation of reaction centers may be due to the inefficiency of the repair system that replaces damaged reaction centers with newly synthesized D1 and D2 proteins, restoring in this way the normal PSII activity (Ohad et al., 1984; Aro et al., 1993).

According to previously published data, the increase of the initial Chl a fluorescence at open centers ($F_0$) may also result from the rearrangements of LHCII–PSII and a partial aggregation of LHCII ( Lazár, 1999). Indeed, the analysis of LHCII sub-complexes in thylakoid membranes isolated from the cultures exposed to UVB shows that the increase in the oligomeric/monomeric fraction ratio upon UVB radiation

is higher in LL than in HL (Fig. 14C). In turn, this increase results in a higher rate of inactivation of the reaction centers possessing a larger antenna (α-centers) (Fig. 9), which suggests the weak functional stability of these centers during exposure to UVB. In addition to these changes in the structure and function of reaction centers, conformational re-adjustments of PSII units within the thylakoid membranes can also take place upon UVB irradiation (Masi and Melis, 1992).

The investigation of antenna size showed that in HL+UVB condition the size of LHCII (as indicated by the oligomeric/monomeric ratio) is much smaller than in LL+UVB conditions (Fig. 14). This reduction of the UVB effect on the LHCII size associated with the low rate of reaction center inactivation (Fig. 5B) denotes that HL acts antagonistically to UVB radiation in the regulation of the photosynthetic behavior. In contrast, LL exerts a synergistic action with UVB radiation, resulting in the exacerbation of UVB effects on the photosynthetic apparatus (Figs. 3, 5-6). Thus, HL intensity assures some protection to UVB stress by maintaining the reaction centers in a functional state. This means that HL has allowed such re-adjustments of the photosynthetic apparatus that excitation pressure on PSII is reduced (Fig. 6E) and a greater proportion of $Q_A$ are kept in the oxidized state (Fig. 6C). The results obtained suggest that these photosynthetic re-adjustments are reflected, in part, by severa1 phenomena. First, the reduction in the Chl amount per cell, a higher Chl a/b ratio (Fig. 11A-B), and a decrease of the LHCII oligomers/monomers (Fig. 14A-C) reduces the probability of light absorption. Second, the increased photosynthetic capacity of HL-incubated cultures as compared to that of LL-incubated cultures (Fig. 10) also decreases the excitation pressure on PSII through concomitant increases in the flux of electrons through PSII and photochemical quenching (Fig. 6B-C). In turn, the reduction of antenna size leads to a decrease in the rate of energy dissipation as compared to LL or D (Fig. 5D). Ultimately, all these changes resulted in the improvement of the efficiency of the photochemical utilization of the absorbed energy as it is expressed in HL (Figs.5A, 6A) and higher capacity for oxygen evolution as in D or LL (Fig. 10).

According to recent data, the overall rate of photosynthesis in cultures exposed to UVB is also limited by the turnover of PSII (Aro et al., 1990; Barbato et al., 1999, 2000). The addition of UVB makes light damage more efficient even under light limiting conditions by altering the function (ability to generate stable charge separation) but more important the physical structure of reaction centers (where the eventual result is the

decrease in the probability of PQ reducing). This might explain the highest loss in PSII functionality observed in LL than in D condition (Fig. 5). It is certain that in light optimal conditions (e.g. HL), the amplitude of damage induced by UVB is diminished (Fig. 10). It is well established that PAR intensity influences a series of responses to UVB stress. In this context, Mirecki and Teramura (1984) found that soybean leaves that are simultaneously irradiated with UVB and high intensities of visible light were resistant to UVB damage, whereas leaves irradiated with UVB and low intensities of visible light were sensitive. Another kind of evidence that emerges is the fact that a previous study (Cen and Bornman, 1990) with *Phaseolus vulgaris* plants exposed to different light regimes and UVB radiation, showed that light intensity is a factor affecting the range of UVB effects in plants. Plants grown under high light conditions were most resistant to UVB radiation, whereas low light conditions enhanced the responses exhibited to UV radiation. Plants grown in medium light in general showed an intermediate response. Other researchers also reported that supplementary UVB light under high PAR shows deleterious effects with field and glasshouse plants being less sensitive to enhanced UVB radiation, when compared to plants raised under lower irradiance (Strid et al., 1990). In contrast, Warner and Caldwell (1983), also working with soybean leaves, showed an increased inhibitory effect of UVB light on photosynthesis when high but non-photoinhibitory visible light was present during the UVB treatment. These results indicate the complexity of photoinhibition in the presence of visible and UV light and suggest the involvement of protective or repair mechanisms that can be modulated by either of the two light qualities. However, higher levels of visible light may also contribute to protection by providing additional substrates through increases in photosynthesis for the repair or replacement of damaged organelles or tissues (Adamse and Britz, 1992).

The higher accumulation of Chl upon LL+UVB mixture (Fig. 11A), can also account for the increase in the total LHCII amount, which means an increased accumulation of LHCII sub-complexes, besides the effects exerted on its oligomerization state (Fig. 14C-D). These responses constitute part of the mechanism which results in the synergistic effect of LL in the amplification of UVB damage. The results obtained in low light cultures are consistent with the finding that the increase of Chl level in conditions in which photosynthesis decreased implies that UVB radiation is not a limiting factor for Chl biosynthesis (Mirecki and Teramura, 1984). Excepting the LL cultures, the Chl

content in HL and D conditions was not significantly altered by UVB radiation (Fig. 11A). Data sets from literature concerning the accumulation of Chl under enhanced UVB irradiation are contradictory. However, increases and decreases in photosynthetic pigments have been observed with increased UVB radiation (Day and Vogelmann, 1995; Correia et al., 1999). Mirecki and Teramura (1984) have demonstrated that light intensity conditions in which UVB irradiation is performed exerts a high influence on the Chl pattern. They found that plants exposed to UVB in low light conditions possessed a higher Chl content than the respective control. Also, Chl b concentration was significantly reduced in low light plants irradiated with UVB. Chl a/b ratio is a factor indicative of LHCII antenna size. Since Chl b is exclusively bound to LHCII and I antenna, an increase in this ratio indicates a lower Chl b concentration and consequently a lower antenna size (Anderson et al., 1988). As we expected, in cultures exposed to UVB irradiation the Chl a/b ratio was lowered (Fig. 11B). This confirms our previously mentioned finding that antenna size increased during UVB radiation (Fig. 14C). Oscillations in the Chl a/b ratio under UVB treatment were also observed in studies performed by other investigators. Vu et al. (1981) reported that Chl a/b ratios decreased with increasing UVB irradiance in soybean but increased in pea at high UV irradiance (Vu et al., 1984). Tevini et al. (1981) concluded that UVB irradiance inhibited the biosynthesis of Chl b more than a, since Chl a/b ratios increased in several species. In contrast, Teramura et al. (1980) reported that no important change occurs in Chl a/b during UVB irradiation. The fluctuations of Chl a/b ratios in irradiated *Scenedesmus* cultures, although not very significant quantitatively are correlated to the oscillations in antenna size. The changes that occurred in Chl content, as well as in Chl a/b ratio indicate that cultures may adapt to the conditions applied during experiments.

Light intensity exerts a regulatory role not only on the Chl, but also on the Car accumulation (Fig. 12). DIfferent studies have shown that Car serve a protective function against UVB (White and Jahnke, 2002; Tevini, 2004). The efficacy of Car in protecting the photosystems is likely due to their function as efficient quenchers of high energy shortwave radiation. The mechanism by which this is accomplished was first proposed to involve a photochemical state change of singlet oxygen to triplet form by interaction with Car, removing the potentially dangerous oxygen radicals produced in photooxidative processes (Krinsky, 1993). Amongst Car, mainly xanthophylls absorb the shortest wavelength radiations within the light-harvesting complexes. Besides the

accumulation of β-carotene and lutein that are correlated to the increasing in antenna size (Bishop, 1996) and therefore can be considered as specific responses to UVB we found a differential accumulation of xanthophylls relative to the illumination conditions used as background in UVB treatments. For instance, HL+UVB stimulates the accumulation of β-xanthophylls (Vx, Ax, Zx) (Fig. 12) inducing a higher increase in the xanthophyll pool than in LL+UVB conditions (Fig. 13). Previous data have shown that plant exposure to sunlight with a high UVB portion may result in increased susceptibility to photoinhibition by visible light and thus amplifying the separate, potentially deleterious actions of UVB and visible light (Pfündel et al., 1992). Indeed, in HL+UVB conditions the xanthophyll cycle (conversion of Vx to Zx) seems to be inhibited, but the increasing in the xanthopyll pool can be one of the protection mechanisms by which HL confers some degree of protection against UVB stress. Also, LL+UVB condition stimulated the increase in the xanthophyll pool without contributing to the activation of xanthophyll cycle (Fig. 13). It is believed that qN is tightly related to the conversion of Vx into Zx and that the Zx-associated qN occurs in the LHCII complexes, since xanthophylls are predominantly organised in the LHCII (Siefermann-Harms, 1985; Bassi et al., 1993). The data presented here show that the high increase in the thermal dissipation of the excess excitation energy (Fig. 5D) due to the enlargement of LHCII size (Figs. 5C, 14C) occurred, although the xanthophyll cycle does not operate upon UVB irradiation (Fig. 13). The hypothesis that Zx and Ax do not directly quench Chl a* molecules but that they facilitate the aggregation of LHCII and as a result the enhancement of the non-photochemical quenching (Horton et al. 1996) is not applicable here. The increases found in LHCII size and energy dissipation under UVB exposure occur in our experiments without Vx conversion. Whether or not xanthophylls contribute to the dissipation of excess energy, the fact is indubitable that upon UVB radiation, the LHCII and inactivated reaction centers contribute to the Chl fluorescence quenching.

Until now, the effect of UVB irradiation on the associated light harvesting complexes is equivocal, since inconsistent results have been reported on the relative changes in the constituent photosynthetic pigments, Chl and Car. However, the greatest effect of UVB radiation on photosynthetic pigments and other plant responses has been observed in LL, whereas plants grown under HL conditions are less affected by UVB, a result that may be attributed to photoprotection or photorepair induced by visible light (Warner and Caldwell, 1983; Mirecki and Teramura, 1984; Cen and Bornman, 1990).

It seems that the regulation of the photosynthetic apparatus responses to UVB by PAR is made through changes in the thylakoid-associated polyamine pattern (Fig. 17). The Put/Spm ratio is more rapidly decreased in LL+UVB than in HL+UVB conditions (Fig. 18B) and this effect is proportional with the magnitude of the effect exerted by PAR on functional antenna size (Fig. 5C), proving once more that the sensitivity of the photosynthetic apparatus to UVB is modulated by PAR through adjustments of antenna size. The UVB-induced decrease in the Put/Spm ratio in thylakoids is also influenced by PAR intensity. LL increased the thyalkoid-associated Spm in detriment of Put, whilst HL keep Put unchanged but increased Spm (Fig. 17). This might constitute another protective effect of HL since it was found that Put confers a higher degree of protection against abiotic stresses. This result is in agreement with other authors who show that under photoinhibitory conditions the Put level increases (Dondini et al., 2003). In contrast, the well-observed increase in the Spm amount bound to thylakoid membranes (Fig. 17) seems to be exclusively a UVB induced effect that is related to the increase in the antenna size. The intracellular accumulation of polyamines in PAR+UVB conditions is also consistent with the data reported by other investigators (Kramer et al., 1991; Krizek et al., 1993). Contrary to their opinion that the photosynthetic apparatus sensitivity to UVB radiation is influenced by the intracellular polyamine content, the data presented here show that the sensitivity to UVB is dependent on the changes in the pattern of thylakoid–associated polyamines. The data obtained for LL+UVB irradiated cultures showed that the decrease in the amount of Put associated to thylakoids (Fig. 17) is followed by a similar decline in the Put/Spm ratio (Fig. 18B). According to Kotzabasis et al. (1999) the decrease of Put/Spm ratio leads to changes in structure and function of the photosynthetic apparatus similar to those observed in low light adapted cultures, e.g. an enlargement of the LHCII accompanied by a decrease of the number of reaction centers per unit areas and Chl a/b ratio. Consistent with this finding are the data obtained for UVB irradiated cultures, where the changes occurring in the Chl a/b ratio, LHCII size and density of active reaction centers together with the decrease of the Put/Spm ratio suggests that UVB induced changes similar to those found in low light adaptation. The fact that this behaviour exhibited by the photosynthetic apparatus could have the significance of an adaptive response to UVB radiation is sustained by the data obtained for recovery, when inverse changes as those found under UVB irradiation are occurring.

In addition, the lowering of Put/Spm ratio may contribute to protection of reaction center proteins, as it was suggested by Besford et al. (1993).

The differences of magnitude in the culture response to UVB are determined by PAR intensity. In LL conditions, the response of cultures to UVB is more pronounced than in HL, suggesting that low PAR intensities act synergistically to UVB radiation, due to the supplementary enlargement of LHCII size that is known to be a specific LL effect. In contrast, high PAR intensities act antagonistically to UVB radiation, because the LHCII size remains smaller and this attenuates the intensity of responses as they are exhibited in D+UVB or LL+UVB conditions. The pattern of changes in Put and Spm biosynthesis, as indicated by the above data, suggests that cultures are capable of adapting their polyamine levels in a way to resist to UVB radiation. It seems that the increase in Spm-associated to thylakoids is the primary mechanism induced in the photosynthetic apparatus by UVB radiation. In the support of this hypothesis come the data obtained for different environmental stress situations such as ozone (Navakoudis et al., 2003), low temperature (Sfakianakis et al., 2006) and UVB (Sfichi et al., 2004; Lütz et al., 2005), which show that abiotic stresses induce the lowering of Put/Spm ratio with a subsequent increase in the functional antenna size and energy dissipation. Taking into consideration that these environmental stresses determine an increase in the excitation pressure of PSII, the reduction of Put/Spm ratio due to Spm increasing plays a protective role, since it contributes to the minimizing of the excitation pressure by enhancing the thermal dissipation of excess energy through an increased antenna size.

In summary, the results presented here support the hypothesis that, due to thermodynamic constraints, *S. obliquus* cultures exposed to UVB adjust the photosynthetic apparatus in response to the excitation pressure on PSII imposed by UVB. The thylakoid-associated polyamines may act as a signal triggering structural and/or functional adjustments of the LHCII and PSII reaction centers (Figs. 14C, 18C). Taking into account that the PAR/UVB ratio used in HL+UVB experiments is quite similar to that found in visible light (Fig. 1), one can easily affirm that, in the natural environment, the photosynthetic apparatus can face out the UVB stress, due to its capability to adjust properly (structurally and functionally) to the environmental stress conditions.

3. Light dependency of the recovery potential of the photosynthetic apparatus affected by UVB radiation

The recovery potential of the photosynthetic apparatus after the cessation of UVB treatment was clearly expressed in the cultures incubated in light conditions. An inverse cascade of responses as those induced by UVB assured the reversion of the photosynthetic apparatus to the initial state. Specifically, the cessation of UVB treatment was followed by the reoxidation of PQ pool (Fig. 6C), due to the re-adjustment of the thylakoids-associated Put/Spm ratio and therefore of the reconstruction of the antenna and the reaction centers (Figs. 5C, 14, 18B). The transition of the LHCII from the high quenched state adopted upon UVB radiation to the low one, due to transition of oligomers to monomers (Fig. 14) resulted in the limitation of light energy losses (Fig. 5D) and the enhancing of electron transport (Figs. 6, 7). Due to this activation, more reaction centers become active (Figs. 5B, 9C) assuring higher efficiency of quantum yields (Figs. 5A, 6A**)** and subsequently higher rates of oxygen evolution (Fig. 10). As a consequence, the excitation pressure on PSII decreased (Fig. 6E), and this is accompanied by the increase of the thylakoidal Put/Spm ratio (Fig. 18B). All these chains of reactions are working only in light conditions (Figs. 5-7). A photosynthetic apparatus incubated in darkness possesses no recovery potential (Figs. 5, 10).

It appears that the absence of PAR blocks the restoration of PSII activity due to the inability to repair the damaged reaction centers. Recently, it was found that a 20 kDa C-terminal fragment of D1 protein generated during irradiation with UVB light was stable when plants were incubated in the dark, but was degraded when plants were incubated in visible light (Bergo et al., 2003). Recovery in darkness did not occur, probably due to the dephosphorylation of reaction center proteins leading to their degradation (Aro et al., 1990). It was shown that UVB promotes dephosphorylation of thylakoid phosphoproteins and for reaction centers proteins (D1 and D2) this is paralleled by protein degradation (Barbato et al., 1999). As it has been demonstrated, the phosphorylation of LHCII polypeptides regulate the energy distribution between PSI and PSII (Allen et al., 1981), while the phosphorylation of D1 protein from the reaction center is suggested to play a role in the regulation of its light-induced turnover (Elich et al., 1992) possibly by preventing the degradation of the phosphorylated damaged form of the protein (Rintamäki et al., 1995). Since the signal for phosphorylation is associated with the reduction of PQ pool level (Allen et al., 1981), it was hypothesized that UVB

light interferes with this regulation, as it induces the degradation of PQ moiety (Melis et al., 1992; Barbato et al., 1995). Several data suggested that the effect of UVB light in the absence of visible light is similar to a dark adaptation with the deactivation of kinase activity. An experiment performed by Barbato et al. (1999) demonstrated that dark-induced dephosphorylation occurs more slowly than that observed under UVB. Moreover, while in the dark LHCII is the complex first dephosphorylated (Bennet, 1980), CP 43 is first dephosphorylated in UVB (Rajagopal et al., 2000). All these data show that phosphorylated D1 protein is not protected against damage induced by UVB light, but in light conditions, is not further degraded by protease activity (Barbato et al., 1999). These results underline the importance of light in the recovery of changes induced by UVB treatment. Similar to our results, Stapleton et al. (1997) observed in maize that recovery was fully accomplished in light while it was absent in darkness. Other data indicate that recovery from the UVB treatment required the lowering of the D1 turnover as compared to the radiation stress (Olsson et al., 2000). Recovery in low light conditions after exposure to sunlight including UVB light has been shown to occur very slowly, probably due to low capacity to restore PSII activity via protein degradation and resynthesis (Aro et al., 1993). Studies that applied artificial UVB (Friso et al., 1994; Jansen et al., 1998) suggest that both the D1 and D2 proteins of the PSII reaction center might need replacing for recovery.

The recovery of changes induced by UVB radiation in *Scenedesmus* cultures shows that the repair system is able to regenerate and maintain the normal population of functional PSII complexes. Light influences the recovery ability, but its intensity is not as significant for the recovery potential as for the determination of sensitivity degree upon UVB exposure. A beneficial influence of LL should be noted, since the higher damage occurring in LL was recovered to the same level with the smaller one induced in HL conditions (Figs. 5, 10) by restoration of α-centers functionality (Fig. 9A) and density of active reaction centers (Figs. 5B, 9C). According to Strasser et al. (1995), the increase in the number of PSII reaction centers (Figs. 5B, 9C) may reflect the necessity for an enhanced electron transport capacity of PSII in the scope of the UVB induced damage repair. To increase the number of active PSII reaction centers and at the same time to maintain a balance of excitation energy between the two photosystems requires the decrease in the LHCII oligomerization state, fact that occurred so in LL, as well as, in HL (Fig. 14).

## *CONCLUSIONS*

Summarizing, the conclusions drawn from the data presented in Results and Discussion- *Chapter 1* can be described as followed:

1. UVB damage results from the cumulative fluences of the changes induced in the molecular structure, conformation and function of the photosynthetic apparatus.

2. The behavior adopted by the photosynthetic apparatus during UVB exposure consists in a chain of reactions initiated by an increase in the excitation pressure on PSII and regulated by means of LHCII antenna mimicking LL-photoadaptation.

3. The sensitivity of the photosynthetic apparatus to UVB radiation is influenced by visible light intensity. Low light intensities increase the susceptibility of the photosynthetic apparatus to UVB damage, whilst high light intensities confer certain degree of protection, making the photosynthetic apparatus more tolerant to UVB stress. The synergistic action of LL or the antagonistic action of HL with UVB radiation is related to the changes of the thylakoidal Put/Spm ratio that adjust the oligomerization status of LHCII.

4. The potential of the photosynthetic apparatus to recover from changes induced by UVB is strictly expressed only in light conditions and characterized by inverse responses from those expressed under UVB treatment. In other terms, the response under UVB mimics LL-photoadaptation, whereas recovery simulates HL-photoadaptation.

5. In the context of the different ozone depletion scenarios, predictions of increase in the amount of UVB radiation reaching the Earth's are a common theme. The data presented here demonstrate that the photosynthetic apparatus possesses the tools and mechanisms to face out the stressor by adjusting the balance between energy absorption and dissipation.

The results obtained from this study suggest that the LHCII is an important determinant of the photosynthetic apparatus sensitivity to UVB radiation. To gain more insights on its role, a comparative study of the photosynthetic behavior adopted, during UVB exposure, by a *wt* photosynthetic apparatus (possessing LHCII antenna) and a *wt-lhc* mutant one (without LHCII antenna) was made (*see* Results and Discussion-*Chapter II*).

# *CHAPTER II*

## THE KEY ROLE OF LHCII IN BALANCING TOLERANCE AND SENSITIVITY OF THE PHOTOSYNTHETIC APPARATUS AGAINST UVB

## *RESULTS*

Data presented in Results and Discussion-*Chapter 1* reveal that the LHCII is the key determinant of the degree of photosynthetic apparatus sensitivity to UVB radiation. By modulation of LHCII size, PAR can alleviate the UVB impact on the photosynthetic apparatus, even though UVB radiation induces an increase in antenna size followed by an entire cascade of responses which, ultimately, alters the photosynthetic capacity. It is widely accepted that the response of the photosynthetic apparatus to environmental factors is not only influenced by the quantity, but also by the spectral quality of visible light (Walters, 2005). Changes in spectral quality (without altering the total incident light) lead to increases in the levels of PSII (and LHCII) relative to PSI, with negligible changes in photosynthetic capacity (Chow et al., 1990; Kim et al., 1993; Walters and Horton, 1994). Under conditions of natural shade where light reaching the plant is enriched in far-red wavelengths preferentially absorbed by PSI, increases in the relative level of PSII are believed to ensure that the supply of electrons from PSII is sufficient to keep pace with the rate of excitation of PSI so that light reaching PSI is used efficiently. Conversely, in unshaded conditions a decreased PSII/PSI ratio is again believed to ensure that the rates of PSI and PSII excitation are balanced, so that absorbed light is used efficiently (Walters, 2005). In green algae, low light intensities can be mimicked by monochromatic blue light, whereas red light can induce HL-adaptive responses (Hoffmann and Senger, 1988).

In the literature, there are few studies highlighting the role of LHCII in the photosynthetic apparatus sensitivity to environmental stresses (Navakoudis et al., 2003; Sfakianakis et al., 2006) and no study focused on the regulation of LHCII by PAR in UVB stress conditions. In this context, the role of LHCII in the photosynthetic apparatus response to UVB radiation was assessed through a comparative study of the photosynthetic behavior adopted to UVB radiation by two strains of *Scenedesmus obliquus*, namely the *wt* (possessing the LHCII antenna) and the *wt-lhc* mutant (without the LHCII antenna). In order to ascertain the primary photoreceptors that regulate the response to UVB radiation, through modulation of the LHCII size,

action spectra (from 410 to 730 nm) of the photosynthetic apparatus bioenergetics in UVB stress conditions, were constructed for both *wt* and *wt-lhc* cultures.

## 1. THE ROLE OF LHCII IN THE PHOTOSYNTHETIC APPARATUS RESPONSE TO UVB RADIATION

The mutant *wt-lhc* has only Chl a and the pigment-protein complexes, CPI (PSI) and CPa (PSII) and no Chl b and light harvesting system. During adaptation to LL or HL, the mutant cultures exhibited, phenotypically, the same adaptational behavior, as the *wt* cultures, although changes in the molecular structure were different. A loss in quantum efficiency under LL intensities was reported for the *wt-lhc* mutant of *S. obliquus* (Bishop et al., 1989). This demonstrates the importance of LHCII in the trapping the excitation energy in the photosynthetic apparatus and, therefore, it is reasonable to investigate the mutant behavior in UVB stress conditions. Furthermore, the finding that polyamines are associated to LHCII apoproteins (Del Duca et al., 1994) give more significance to this type study, since it seems that the primary response to UVB is represented by changes in the polyamine pattern followed by changes in the LHCII size. The same experimental procedure consisting in 3 h of UVB exposure to 0.42 mW $cm^{-2}$ and 4 h of additional recovery in the low light conditions was applied (*see* Material and Methods).

### 1.1. LHCII IMPORTANCE IN PSII FUNCTIONALITY UNDER UVB TREATMENT AND RECOVERY

The functionality of PSII was investigated using polarographic and fluorescence induction measurements. These measurements are known to be sensitive, rapid, non-invasive and reliable methods for *in vivo* assessment of the photosynthetic apparatus response to abiotic factors. Moreover, the analysis of the yield of variable fluorescence can give information on the action sites of UVB. This is because it is a measure of the PSII primary photochemistry and associated electron transport intensively used in the investigation of molecular targets in PSII of different environmental stresses, such as high light (Golan et al., 2006), low temperature (Ivanov, 2006), heat (Srivastava et al., 1997), ozone (Navakoudis et al., 2003), UVB (Sfichi et al., 2004; Lütz et al., 2005) etc.

#### 1.1.1. Changes in photosynthetic apparatus bioenergetics in the *wt* and *wt-lhc* cultures

The investigation of O-J-I-P transients in control cultures (as measured prior to UVB irradiation) indicates that in the *wt-lhc* culture the reduction of PQ is faster than in

the *wt* culture. Upon UVB radiation, the fluorescence yield at the J-P phase significantly decreased (Fig. 1). In both cultures, the typical O-J-I-P transients were progressively transformed into a smoother O-J-P transient, during the exposure to UVB, due to progressive loss of fluorescence yield at the J, I and P steps.

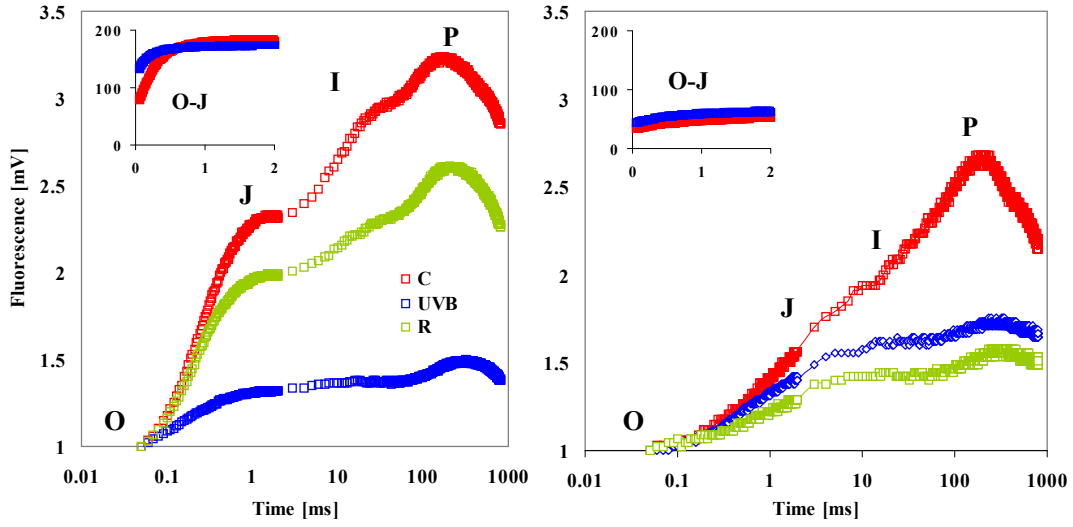


**FIGURE 1.** Changes in the shape of O-J-I-P transients in *wt* (*left*) and *wt-lhc* (*right*) cultures, prior to UVB irradiation (C), after 3h of UVB irradiation (UVB) and after 4 h of additional recovery (R). Curves are normalized at $F_0$ and time given on a logarithmic scale. Insets represent the fluorescence from O (50μs) to J (2ms), on linear time scale.

This strong fluorescence quenching of the J-I phase can be explained by the decreased rate of electron donation from the OEC to the PSII centers (Schreiber and Neubauer, 1987). Although in the *wt*+UVB culture, the J-P phase suppression is strong, the fluorescence yield recovered after the cessation of UVB treatment. In contrast, there is an irreversible lost of fluorescence yield in *wt-lhc*, demonstrating that the presence of LHCII is vital for the recovery of PSII damage induced by UVB (Fig. 1).

To investigate the reduction and oxidation of $Q_A$, the sample cultures were poisoned with 100 μM DCMU. It is well known that it blocks the electron flow from $Q_A$ to the secondary quinone acceptor of PSII, $Q_B$ and thereby the re-oxidation of the $Q_A^-$ is prevented (Melis and Homann, 1975). With its binding in the $Q_B$ pocket of the D1 protein, DCMU also curtails the reduction of the PQ pool allowing the accumulation of oxidized PQ. In this way it is possible to estimate if the quenching of Fm is in totality resulted from the PSII damage or if the oxidized PQ plays some role in this effect. In the last case the addition of DCMU would lower the Fm parameter. In both UVB-irradiated

cultures, the Fm level in the presence of DCMU was found to be almost similar to the Fm level measured in the absence of DCMU (Fig. 2).

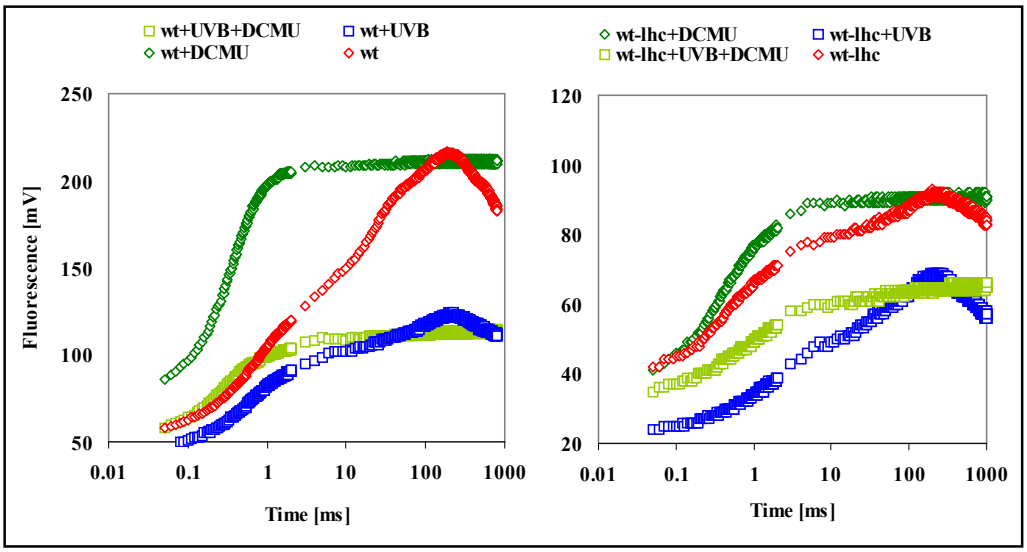


**FIGURE 2.** Kinetics of fluorescence transients in *wt* (*left*) and *wt-lhc* (*right*) culture samples incubated or not with 100 µM DCMU, for 10 min in dark, prior to UVB (*wt* or *wt-lhc*) and after 3 h UVB irradiation treatments (*wt*+UVB; *wt-lhc*+UVB). *Green curve*: *wt*+DCMU; *wt-lhc*+DCMU; *Red curve: wt*; *wt-lhc*; *Blue curve*: *wt*+UVB; *wt-lhc*+UVB; *Green light curve*: *wt*+UVB+DCMU; *wt-lhc*+UVB+DCMU

It was reported that upon UVB irradiation several centers are resistant to DCMU. This is due to the alteration of the $Q_B$ pocket by UVB (Vass et al., 1996) so that DCMU can become less efficient. In order to prevent the possible artifacts due to the inefficiency of DCMU treatment in UVB-treated samples, the maximal net photosynthetic rate was measured in both *wt* and *wt-lhc* cultures and was checked in the presence and in the absence of DCMU, prior and post-UVB treatments. The results indicated that DCMU was effective in the inhibition of the photosynthesis in both control and UVB irradiated samples (data not shown). Thus, the Fm measured in the samples treated with DCMU as compared to the untreated ones shows the full reduction of PQ pool by UVB in both *wt* and *wt-lhc* cultures. The rise of fluorescence yield from O to P step was rapid and attained its maximum at the J-step (Fig. 2) indicating the immediate closure of all PSII centers. It can be seen that in the absence of DCMU, UVB also determined the closure of all reaction centers. This demonstrates that both DCMU and UVB induced the inhibition of electron transport to $Q_B$. From the fluorescence data shown in Fig. 2, the amount of $Q_B$ non-reducing centers was calculated (Fig. 3).

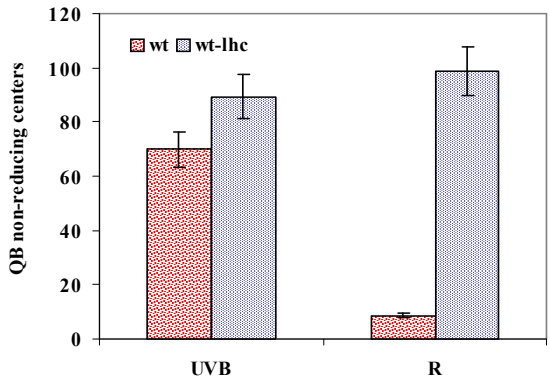


**FIGURE 3.** UVB effect on the amount of $Q_B$ non-reducing centers in *wt* and *wt-lhc* cultures, after 3 h of UVB irradiation and additional 4 h of incubation in the initial light conditions without UVB for recovery (R). The values are given in % from initial control values.

In the absence of LHCII, the inactivation of reaction centers induced by UVB was higher in *wt-lhc*+UVB culture than in *wt*+UVB one. About 90 % of the reaction centers in *wt-lhc*+UVB culture become unable to perform the reduction of $Q_B$, whereas in *wt*+UVB culture the amount of $Q_B$ non-reducing centers was increased by 70%. After the cessation of UVB treatment the effect obtained is significant. In *wt*-UVB(R) culture, the largest amount of inactivated reaction centers became functional again. In contrast, the reaction centers in *wt-lhc*-UVB(R) culture completely lost their functionality (Fig. 3). The existence of such centers has been proposed as a possible protective mechanism (Krause et al., 1990). The inactivated reaction centers are transformed to heat sinks (Strasser et al., 2000), meaning that they can neither reduce $Q_B$ nor back transfer the excitation energy to the antenna and so they do not contribute to the variable fluorescence. Their fluorescence yield remains constantly low and equal to that of open reaction centers; moreover, they are quickly re-activated as soon as the stress that induced their transformation ceases. Our data showed that their re-activation requires the presence of LHCII.

The inactivated reaction centers are responsible for the increases in the functional antenna size (ABS/RC). As shown in Fig. 4, the functional antenna size is increased more in the presence of LHCII (250% in *wt*) than in its absence (160% in *wt-lhc*). In the mutant cultures this increase resulted from the inactivated reaction centers, whereas in the *wt* the increase in the functional antenna size resulted from both, LHCII increasing and inactivated reaction centers (*see* Results and Discussion-*Chapter I*). This means that the contribution of LHCII to the increase in ABS/RC is about 100%. An increase in the functional antenna is followed by a similar increase in the ratio of total dissipation of excess energy to the amount of active reaction centers (DIo/RC) (Fig. 4).

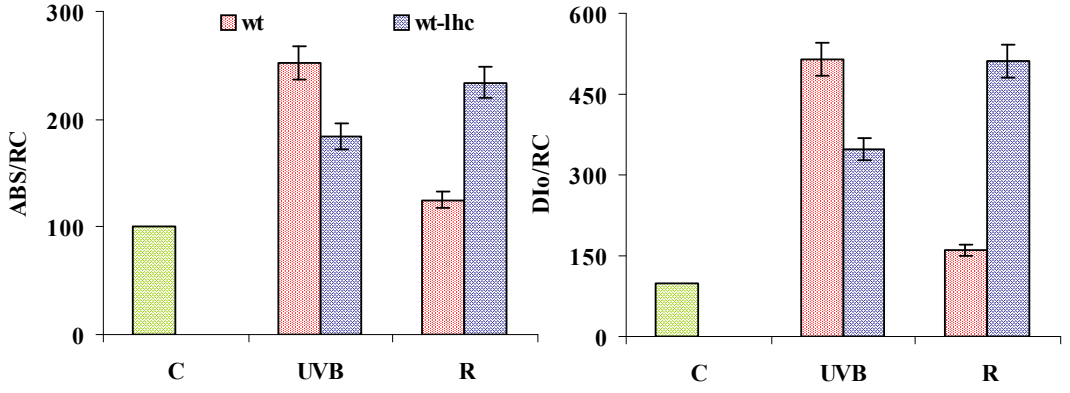


**FIGURE 4.** Changes in the functional antenna size (ABS/RC) and in the rate of excess energy dissipation (DIo/RC), in *wt* and *wt-lhc* cultures, after 3 h of UVB irradiation and additional 4h of recovery (R). The values are given as percentage from the corresponding control (expressed as 100%).

The cessation of the UVB treatment was marked by a decrease in the functional antenna size in *wt*-UVB(R) culture whereas, due to the continuous inactivation of active reaction centers, it increased even more in *wt-lhc*-UVB(R) culture. Consequently, similar changes were registered in the DIo/RC (Fig. 4). The increase in the functional antenna size is related to the higher connectivity (pG) of the photosynthetic units that exists in *wt*+UVB culture as compared to the *wt-lhc*+UVB mutant. In the mutant culture (*wt-lhc*+UVB) the connectivity between the reaction centers is 300% lower than in the *wt*+UVB culture and remained at this level even after 4h of incubation in the absence of UVB (Fig. 5). In contrast, pG recovered after the cessation of UVB treatment in the *wt*+UVB culture.

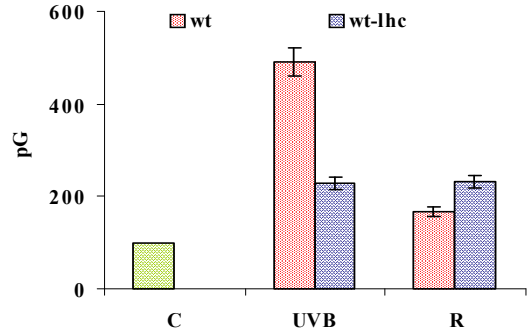


**FIGURE 5.** Changes induced by UVB in the energetic connectivity, expressed as the over-all grouping probability of PSII (pG), in *wt* and *wt-lhc* cultures, after 3 h of UVB irradiation and additional 4h of recovery. The values are given as percentage from the corresponding control (expressed as 100%).

The investigation of fluorescence quenching after light adaptation was used to estimate the function of the photosynthetic apparatus during exposure to continuous light and to gain information about the excitation pressure (1-qP) exerted by UVB on PSII, which is equivalent to the proportion of closed reaction centers. It is closely related to the redox state of PQ so that it is conditioned by the electron flow on PSII.

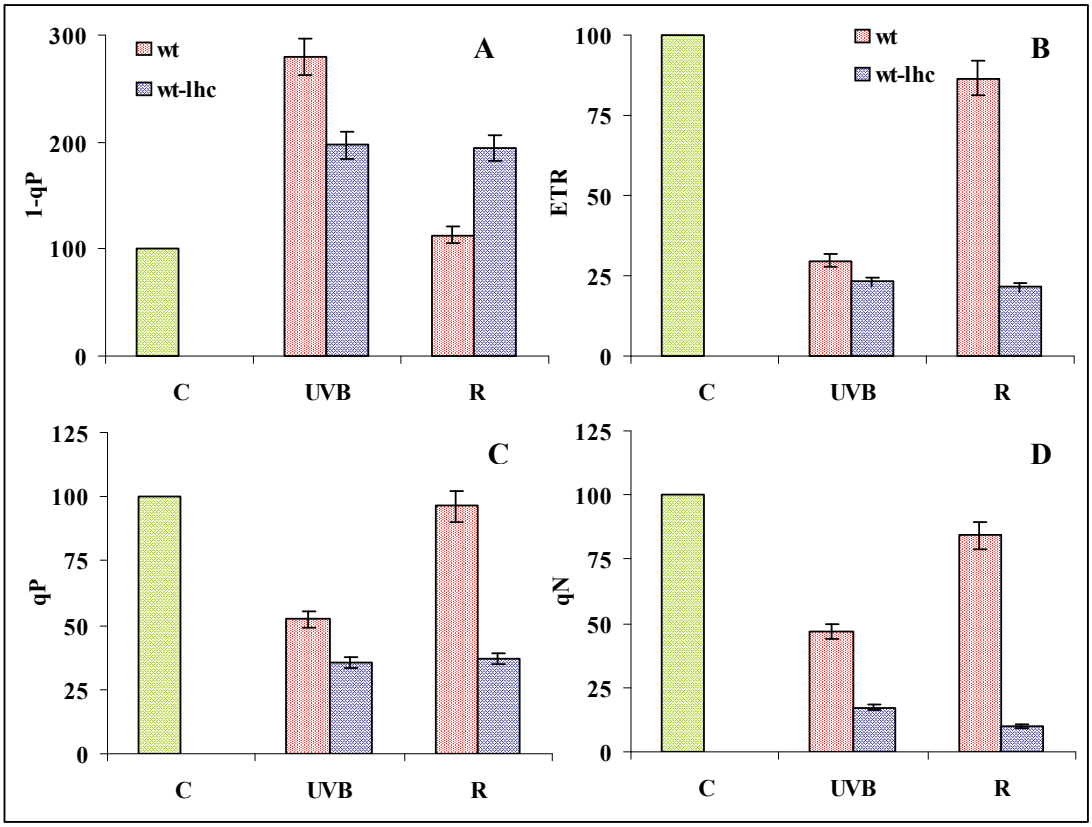


**FIGURE 6.** UVB- induced changes in the (**A**) excitation pressure exerted on PSII (1-qP), (**B**) rate of electron transport in light-adapted state, (**C**) the photochemical (qP) and (**D**) non-photochemical quenching (qN) in *wt* and *wt-lhc* cultures, after 3 h of UVB irradiation and additional 4h of recovery. The values are given as percentage from the corresponding control (expressed as 100%).

Although the ETR (rate of electron transport) in the light-adapted state is almost similarly decreased by UVB in both *wt* and *wt-lhc* cultures (Fig. 6B), the excitation pressure exerted on PSII is about 75% higher in the presence of LHCII (in *wt*+UVB cultures it is 275% over control) than in the mutant (where it reached 200% over control). After the cessation of UVB treatment, both parameters recovered only in *wt*-UVB(R), whereas they were kept at the same UVB-induced level in the *wt-lhc*-UVB(R) culture (Fig. 6A). The photochemical quenching (qP), which is an indicator of the

proportion of open reaction centers, decreased much more in the *wt-lhc*+UVB culture (65%) and lesser in the *wt*+UVB culture where, the presence of LHCII contributed to 15% reduction of the UVB effect on qP (Fig. 6C).

Similarly, the non-photochemical quenching capacity (qN) is net superior when the LHCII is present. In the *wt*+UVB cultures, the values calculated for qN were 50% below the corresponding control, whereas in the *wt-lhc*+UVB culture, qN registered a higher decrease (about 80%). This means that LHCII reduced the UVB effect on qN by 30% (Fig. 6D), also assuring the restoration of quenching capacity to the initial status after the cessation of UVB treatment. These changes indicate that LHCII is also necessary for recovery and the photosynthetic apparatus quenching capacity (photochemical and non-photochemical quenching) is more susceptible to UVB inhibition in the absence of LHCII.

In the *wt-lhc*+UVB culture, the irradiation treatment reduced the maximum quantum yield of the primary photochemistry (Fv/Fm) to 50% of the control, while in *wt*+UVB culture this effect was 10% higher, showing a slight amplificatory effect of the LHCII on the UVB-induced decrease in the PSII photochemistry. After the cessation of UVB treatment, the Fv/Fm showed almost full restoration in the *wt*-UVB(R) culture. In contrast, the *wt-lhc*-UVB(R) mutant did not exhibit the potential to recover from the changes induced by UVB. This fact resulted in an even more pronounced Fv/Fm decline (Fig. 7).

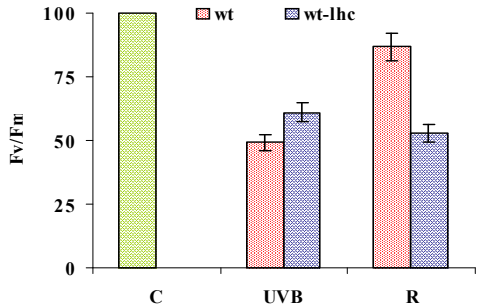


**FIGURE 7.** UVB-induced changes in the maximum quantum yield of PSII (Fv/Fm) in *wt* and *wt-lhc*, after 3 h of UVB irradiation and additional 4h of recovery (R). The values are given as percentage from the corresponding control (expressed as 100%).

An accurate estimation of LHCII contribution to the overall response of the photosynthetic apparatus to UVB requires the investigation of bioenergetics of the two cultures prior to the application of the UVB treatment. For this purpose, several parameters selected on the basis of their susceptibility to UVB (in a *wt* photosynthetic

apparatus), were projected in a radar plot. The reference values were those obtained for the *wt-lhc* culture.

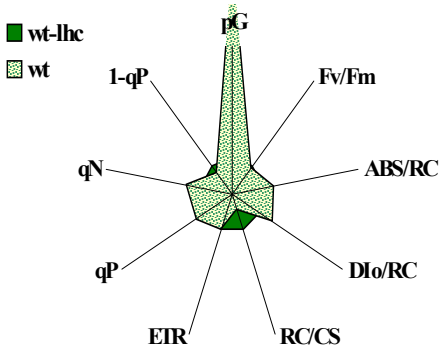


**FIGURE 8.** Radar plots of several parameters and expressions calculated in *wt* and *wt-lhc* cultures, used as control (prior to UVB irradiation). The reference values are of the mutant *wt-lhc.*

As depicted in Fig. 8, the LHCII presence leads to a larger functional antenna (ABS/RC) associated with a higher rate of energy dissipation per active reaction center (DIo/RC). Although the number of active reaction centers (RC/CS) in the *wt* culture is lower than in the *wt-lhc* mutant, the higher energetic connectivity between reaction centers (pG) is accompanied by an increased capacity of photochemical and non-photochemical quenching and the excitation pressure exerted on PSII (1-qP) is lower than in the mutant. Taking together all the parameters, one can suppose that in the *wt* the photosynthetic units are interconnected in such way that assures the function of a low number of active PSII reaction centers with maximum efficiency. Thus, a *wt* photosynthetic apparatus seems to work on the principle "maximal energetic efficiency with minimal material".

### 1.1.2. Changes in the maximal net photosynthetic rate in the *wt* and *wt-lhc* cultures

Polarographical measurements of oxygen evolution upon UVB irradiation and after the cessation of UVB treatment indicate that the role of LHCII is highly important in the recovery of photosynthetic ability, which is affected by UVB radiation. In both cultures, the UVB treatment caused a reduction of about 60% in the maximal net photosynthetic rate and this effect did not seem to be significantly influenced by the LHCII (Fig. 9). After the cessation of UVB treatment, the photosynthetic ability for oxygen evolution recovered about 40% in *wt*, while in *wt-lhc* it declined even more.

Undoubtedly, this certifies that the LHCII is primordial for the re-habilitation of the photosynthetic performance affected by the stress factor (Fig. 9).

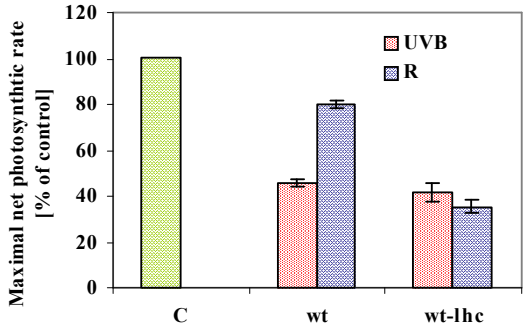


**FIGURE 9.** Maximal net photosynthetic rates in *wt* and *wt-lhc* cultures after 3h of UVB irradiation (UVB) and additional 4h of incubation in conditions without UVB (R). The values are expressed as percentage from the corresponding values measured prior to the treatment (C).

## 1.2. BIOCHEMICAL CHANGES IN THE *wt* AND *wt-lhc* PHOTOSYNTHETIC APPARATUS UPON UVB IRRADIATION AND RECOVERY

### 1.2.1. Quantitative and qualitative changes of intracellular and thylakoid-associated polyamines in the *wt* and *wt-lhc* cultures

Previously, it was shown that the level of intracellular and thylakoid-associated polyamines increased in an UVB-irradiated *wt* photosynthetic apparatus, whereas the Put/Spm ratio decreased. Furthermore, it was found that a reduction in the Put/Spm ratio in thylakoids was accompanied by an increase in the oligomerization state of LHCII (*see* Results and Discussion-*Chapter I*).

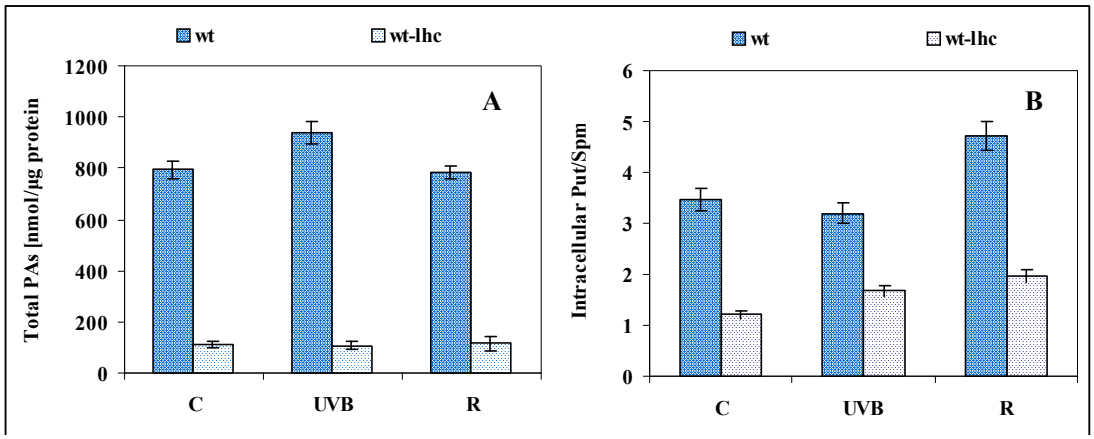


**FIGURE 10.** Differences in the intracellular (**A**) polyamine amount and (**B**) Put/Spm ratio between the *wt* and *wt-lhc* cultures, prior to UVB irradiation (C), after 3 h of UVB irradiation (UVB) and after additional 4 h of incubation without UVB, for recovery (R).

To gain evidence that polyamines are involved in the regulation of LHCII oligomerization status, polyamine-associated to thylakoid membranes, should exhibit a

different pattern of changes in an UVB-irradiated *wt-lhc* photosynthetic apparatus than in the *wt*. Thus, intracellular and thylakoid-associated polyamines from *wt* and *wt-lhc* cultures were extracted, identified and quantified by HPLC.The intracellular polyamine content is about 5 times higher in the *wt* culture than in the *wt-lhc* one (Fig. 10A). Similarly, the intracellular Put/Spm ratio also recorded higher values in the *wt* culture than in the mutant one (Fig. 10B).

Upon UVB radiation, in the *wt-lhc*+UVB mutant there is no change in the polyamine accumulation as it occurred in the *wt*+UVB culture (Fig. 10A), where an increase in the total polyamine amount was observed. UVB radiation induced minor changes in the pattern of intracellular polyamines in both the *wt*+UVB and *wt-lhc*+UVB cultures, although in opposite directions (Fig. 11). Specifically, in the *wt-lhc*+UVB culture the amount of intracellular Put increased to the detriment of Spm and Spd (Fig. 11B), leading to a higher Put/Spm ratio than in *wt*+UVB culture. In the later, there is a higher accumulation of Spd, whereas Put is slightly decreased as compared to the control. Spm is less changed and the Put/Spm ratio is slightly decreased upon UVB irradiation treatment (Fig. 11A).

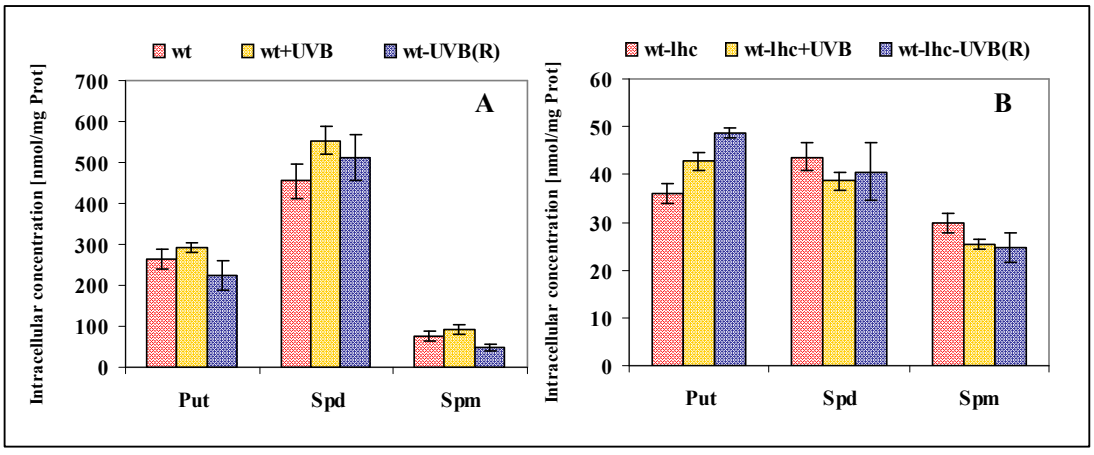


**FIGURE 11.** Differences in the intracellular polyaminecontent between the (**A**) *wt* and (**B**) *wt-lhc* cultures, prior to UVB irradiation (C), after 3 h of UVB irradiation (UVB) and after additional 4 h of incubation without UVB, for recovery (R).

A different situation was found in the quantity and pattern of polyamines associated to thylakoid membranes. Although the amount of total polyamines associated to thylakoids increased upon UVB irradiation in both cultures (Fig. 12A), their pattern was differently changed upon exposure to UVB radiation. Specifically, Put-associated to thylakoids increased in the *wt-lhc* +UVB mutant (by 10%) but not in the *wt*+UVB,

where it decreased to 73% of the corresponding *wt* control (Fig. 12C). In contrast, Spm-associated to thylakoid membranes largely increased in the *wt*+UVB culture (about 200%), while in the mutant culture it decreased by 16%, as compared to the corresponding control (Fig. 12E). These quantitative alterations led to the increase of thylakoidal Put/Spm ratio in the *wt-lhc*+UVB mutant in contrast to the *wt*+UVB culture where the Put/Spm ratio recorded lower values than prior to UVB treatment (Fig. 12B). In both cultures, Spd-associated to thylakoids increased (about 24%) (Fig. 12D).

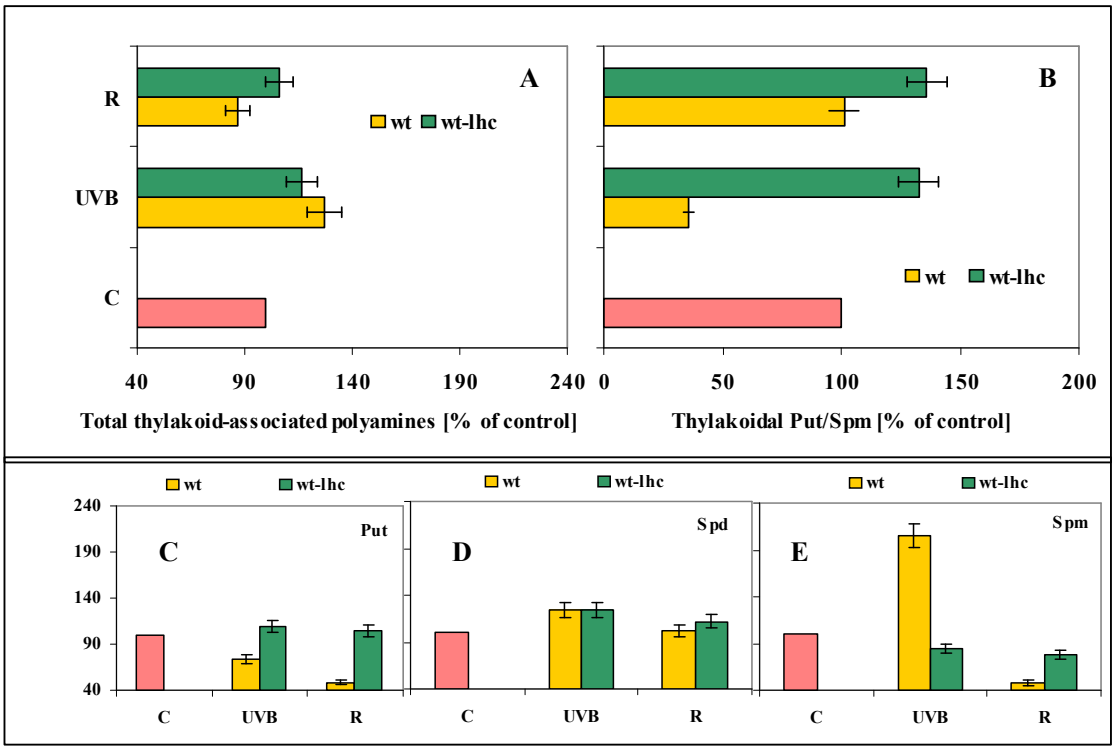


**FIGURE 12.** Quantitative and qualitative differences in the (**A**) total polyamine amount, (**B**) Put/Spm ratio, (**C**) Put, (**D**) Spd and (**E**) Spm in thylakoid membranes isolated from *wt* and *wt-lhc* cultures, after 3h of UVB irradiation (UVB) and after additional 4 h of incubation in conditions without UVB, for recovery (R).The values are given as percentage from the corresponding control cultures (C).

These preceding data suggest that the different pattern of changes in the thylakoid-associated Put and Spm recorded for *wt*+UVB and *wt-lhc*+UVB cultures originate from the different characteristics of the photosynthetic apparatus. A photosynthetic apparatus possessing a LHCII antenna will react to UVB by increasing the amount of Spm-associated to thylakoids, probably to increase the size of antenna; in the absence of LHCII, the photosynthetic apparatus will increase the amount of Put-associated to thylakoids.

### 1.2.2. Changes in the carotenoid content and xanthophyll cycles in the *wt* and *wt-lhc* cultures

Car play several crucial roles in the photosynthetic process namely in light harvesting, photoprotection, protein assembly, regulation of the photosynthetic membrane (Demmig-Adams, 1990). The functional complexity of these compounds, especially their participation in LHCII stabilization or to non-photochemical quenching processes make it reasonable to investigate their pattern and quantity in both *wt* and *wt-lhc* cultures. The quantitative and qualitative determination made prior to the UVB treatment showed that the *wt* culture possesses a higher level of carotenoids than the *wt-lhc* culture. This fact is related to the presence of LHCII (Fig. 13). There are also differences in the distribution of carotenoids; in the *wt* culture the amount of each identified carotenoid decreased in the order: lutein (L) > β-carotene > loroxanthin (Lx) > neoxanthin (Nx) > violaxanthin (Vx) > α-carotene, while in the mutant culture, the accumulation of each carotenoid decreased in the order: α-carotene > L > Vx > Lx > β-carotene > Nx.

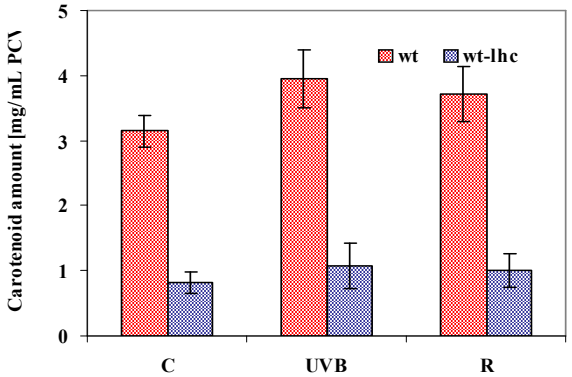


**FIGURE 13.** Total carotenoid amount in *wt* and *wt-lhc* cultures, prior to UVB irradiation (C), after 3h of UVB irradiation (UVB) and an additional 4h time period of incubation in conditions without UVB for recovery (R).

Small amounts of zeaxanthin (Zx) and antehraxanthin (Ax) were also detected in each strain (Fig. 14). The UVB treatment induced an increase in the total carotenoid amount, especially in the *wt*+UVB culture (Fig. 13), in conjunction with alterations in their pattern (Fig. 14). Specifically, there is an increase in the accumulation of β- and α-carotene concurrently to a decrease in the amounts of Nx and Lx. In both cultures; the amounts of L and β-xanthophylls (Vx, Ax and Zx) also increased.

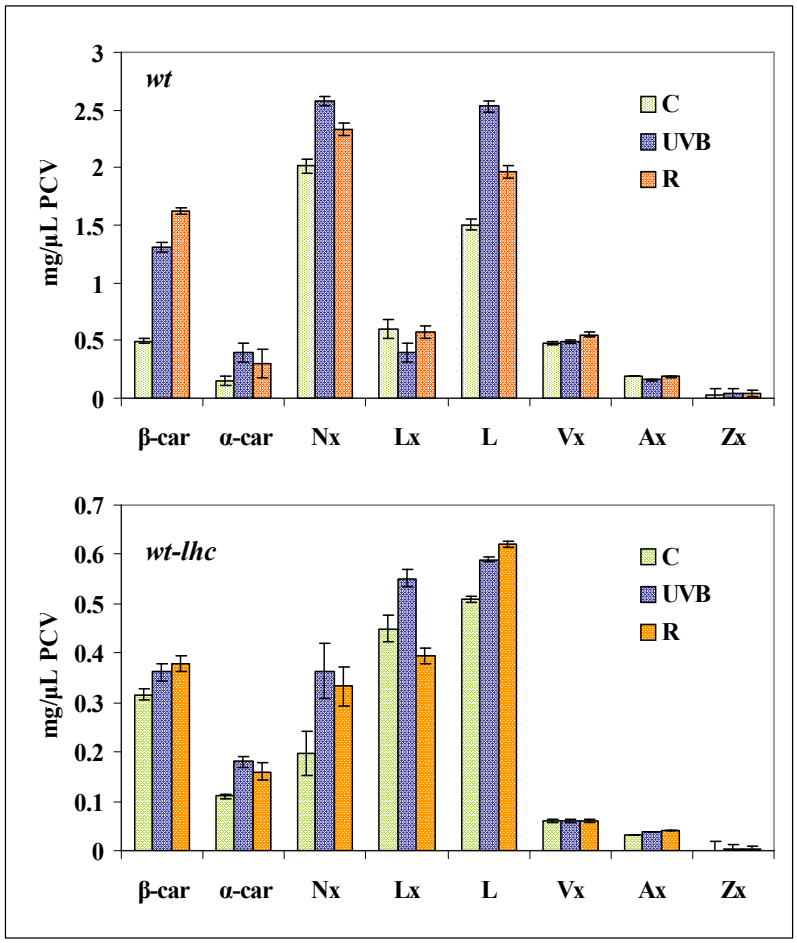


**FIGURE 14.** Characteristics of the carotenoid pattern, analyzed by HPLC, in *wt* and *wt-lhc* cultures before (C), after 3 h of UVB treatment (UVB) and after additional 4 h of recovery (R).

The most abundant carotenoids in the *wt*+UVB culture are L followed by β-carotene and Vx, whereas in *wt-lhc*+UVB culture there is a higher accumulation of α-carotene, followed by L and Vx (Fig. 14). The α- and β-xanthophylls are important compounds in photoprotection that contribute to dissipation of excess excitation energy (Matsubara et al., 2004) and, therefore, the possible alterations induced by UVB in the β-xanthophyll (Vx+Zx+Ax) and α-xanthophyll (Lx+L) accumulation, as well as in the Vx-cycle (de-epoxidation of Vx in Ax and then Zx; Vx/Ax+Zx) and Lx-cycle (interconversion of Lx in lutein; Lx/L) were investigated in both *wt* and *wt-lhc* cultures. The β-xanthophyll pool increased in the *wt-lhc*+UVB mutant, whereas it decreased slightly in the *wt*+UVB culture (Fig. 15A). In contrast, there is a larger increase in the accumulation of α-xanthophylls in the *wt*+UVB culture (Fig. 15C), paralleled by an enhancement of Lx conversion in L (Fig. 15D). In neither the *wt*+UVB culture, nor in

the *wt-lhc* culture, was the Vx cycle operational during exposure to UVB radiation (Fig. 15B).

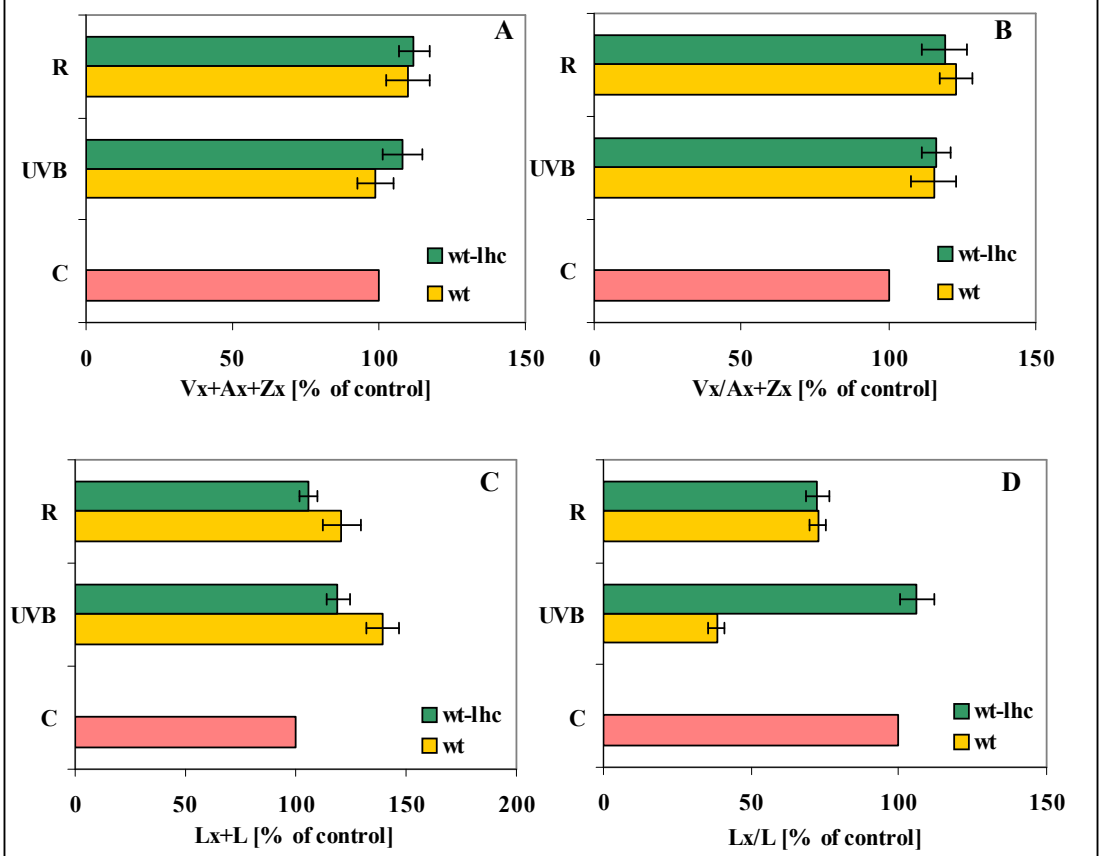


**FIGURE 15.** Qualitative and quantitative changes in the pattern of xanthophylls investigated in *wt* and *wt-lhc* cultures, after 3h of UVB irradiation (UVB) and additional 4 h of recovery in conditions without UVB (R). The values are expressed as percentage from the corresponding control (C) and represent the average of three samples. The β-xanthophyll (Vx+Zx+Ax) pool and the Vx cycle (the conversion of Vx into Zx via Ax, here expressed as Vx/(Zx+Ax) are illustrated in panel **A** and **B**, respectively. The α-xanthophyll (Lx+L) pool and the Lx cycle (the conversion of Lx into L, here expressed as Lx/L) are illustrated in panel **C** and **D**, respectively.

## 2. CHARACTERIZATION OF THE PRIMARY PHOTORECEPTOR(S) REGULATING THROUGH LHCII THE SENSITIVITY AND TOLERANCE OF THE PHOTOSYNTHETIC APPARATUS TO UVB RADIATION

The preceding data demonstrate that the LHCII is the main determinant of the photosynthetic apparatus sensitivity/tolerance to UVB radiation, as well as, the capacity

of recovery from harmful UVB effects. Therefore, the photoreceptors that are responsible for the regulation of photosynthetic apparatus sensitivity to UVB radiation, through changes in the LHCII, were investigated from the action spectra constructed for both *wt* and *wt-lhc* cultures. The experimental design applied is described in Material and Methods 1.3.2.2. Briefly, after 24 h incubation in D conditions, *wt* and *wt-lhc* cultures were exposed for 3h to different wavelengths of monochromatic light (ML) and at white light (WL) of equal intensity (15 μmol $m^{-2}$ $s^{-1}$). The action spectrum of the density of active reaction centers (RC/CS) was made in order to check if the preincubation time (3h) of the cultures to the corresponding monochromatic light was sufficient to induce the photoadaptation of the photosynthetic apparatus to respective light conditions.

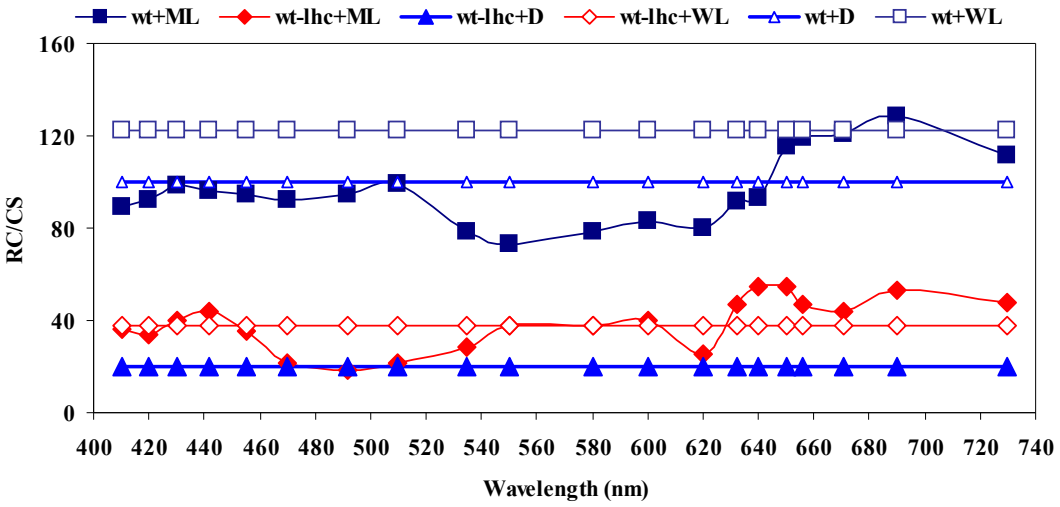


**FIGURE 16.** Action spectra for the density of active reaction centers (RC/CS) in *wt* and *wt-lhc* cultures after 3h of adaptation to monochromatic light (ML) of different wavelengths and WL (WL) of similar intensities (15 μmol $m^{-2}$ $s^{-1}$), as compared to control cultures incubated in dark (D) conditions.

In Fig. 16 it is clearly shown that both cultures exhibited a significant increase in the active reaction center density (RC/CS) in the red light spectral fraction. This is in full agreement with previous findings that low light intensities can be mimicked by monochromatic blue light, whereas red light induce HL-adaptive responses (Hoffmann and Senger, 1988) consisting of an increase in the density of active reaction centers. After photoadaptation to the given light conditions, the cultures were irradiated additionally with 0.42 mW $cm^{-2}$ UVB for 1.5 h and then maintained for an additional 1.5 h in the initial light condition without UVB for recovery.

In order to assess the importance of light quality in the modulation of UVB effects, the response obtained for the cultures incubated in different ML or in WL conditions was compared to that obtained for the *wt* and *wt-lhc* cultures incubated in D during both UVB treatment and recovery periods. Several parameters important to the characterization of photosynthetic apparatus bioenergetics were investigated in both cultures. As a control, the values measured prior to UVB irradiation were used. The processing of data for the quantification of LHCII contribution in the photosynthetic behavior adopted at different irradiation conditions (i.e., ML+UVB, D+UVB, WL+UVB) was made by extracting the values obtained for the *wt-lhc* cultures after 1.5 h of UVB irradiation (expressed as % of the corresponding control) from similarly calculated values of the *wt* cultures. The difference obtained ("Δ") indicates the primary photoreceptors sensing the LHCII, which are responsible for the attenuation or amplification of the UVB effects on the photosynthetic apparatus. Thus, action spectra for UVB sensitivity determined by the LHCII were constructed.

## 2.1. PRIMARY PHOTORECEPTORS ADJUSTING THE PHOTOSYNTHETIC APPARATUS BIOENERGETICS UNDER UVB EXPOSURE

The action spectrum obtained for the maximum quantum yield of PSII (Fv/Fm) shows 3 negative peaks (430, 550 and 656 nm) suggesting that mainly Chl (656/430 nm) and some Car (550 nm) from the LHCII are responsible for the down-regulation of Fv/Fm upon UVB radiation. The negative effect of UVB on the Fv/Fm is reversed at 442, 535 and 620-640 nm. It can be supposed that the two forms of protochlorophyllide (PChlide): free PChlide (620/442 nm) and active PChlide (640/442 nm) (POR-PChlide-NADPH complex; Kotzabasis et al., 1991) could be involved in the reduction of UVB effect on the Fv/Fm. There is no significant effect on the Fv/Fm in the (far)red region of the spectrum, suggesting that neither the reaction centers of PSII nor the PSI are involved in the regulation of UVB effect on the Fv/Fm (Fig. 17A).

The oscillations in the Fv/Fm ratio are induced by the impairment in the PSII activity which leads to the inactivation of active reaction centers (RC/CS). The contribution of photoreceptors to the modulation of reaction center functionality through LHCII is presented in Fig.17B**.**

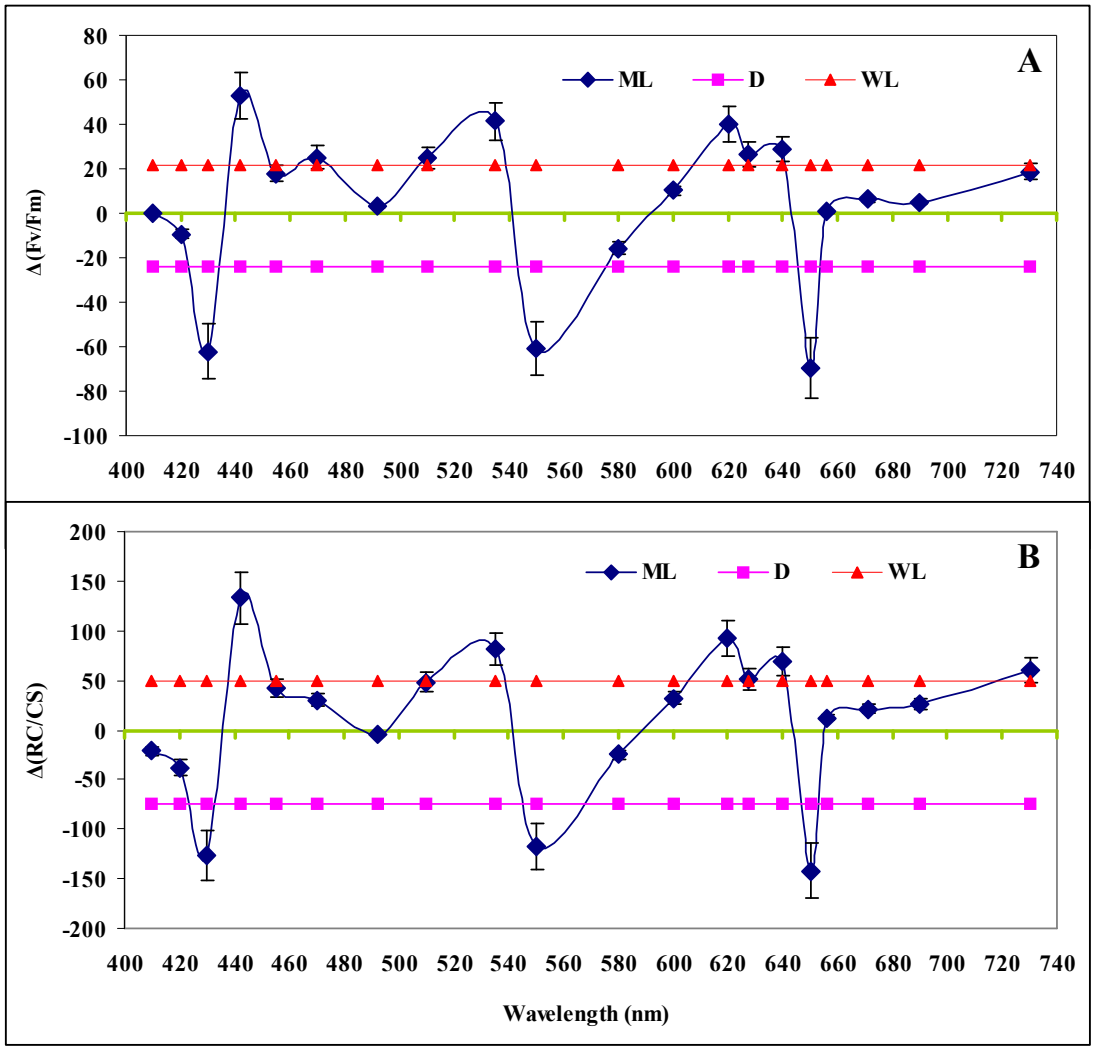


**FIGURE 17.** Action spectrum of the (**A**) maximum quantum yield of PSII (ΔFv/Fm) and (**B**) density of active reaction centers per cross section (ΔRC/CS). The values of Y-axis are calculated as difference between Fv/Fm values obtained for *wt* (as % of control) and those for *wt-lhc* (calculated as % of control) [e.g. Δ=Fv/Fm(*wt*)-Fv/Fm(*wt-lhc*)], which represent the LHCII contribution to the UVB effect. The green line represents the *wt-lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light (ML) of different wavelengths (nm), dark (D) and white light (WL) conditions.

The action spectrum difference (between *wt*+UVB and *wt-lhc*+UVB cultures) of the active reaction centers show again that the PChlide (free and active) absorbed light (620-640 and 430 nm) attenuated the UVB effect, whereas the light absorbed by chlorophylls (Chl) (656/430 nm) as well as an unknown carotenoid (550 nm) lead to an amplification of that effect.

Previously, it was shown that the UVB treatment induced the increase in the functional antenna size (ABS/RC) by inactivating the active reaction centers and by

increasing the oligomerization state of LHCII. Extracting the action spectra values obtained for the ABS/RC in *wt-lhc*+UVB mutant from those obtained for the *wt*+UVB cultures, the action spectrum obtained represents (Fig. 18) in fact regulation by photoreceptors of the LHCII in such manner that the UVB effect is amplified or diminished. The action spectrum for functional antenna size depicts a situation that is in exact opposition compared to that revealed by the action spectrum for the reaction center functionality (Fig. 17B).

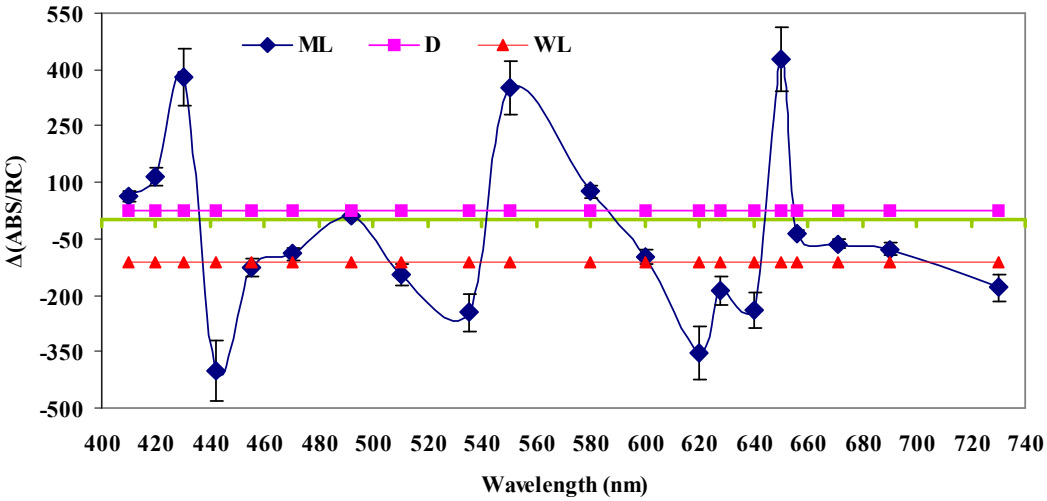


**FIGURE 18.** Action spectrum of the functional antenna size (ΔABS/RC). The green line represents the *wt-lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

Light absorbed by Chl a (656/430 nm) increased the functional antenna and therefore, amplified the UVB effect. On the contrary, the energy absorbed by PChlides (620-640/442 nm) strongly decreased the functional antenna size (Fig. 18) which attenuates the UVB effect on the Fv/Fm and RC/CS parameters (Fig. 17), even in a higher degree than WL. Thus, it can be assumed that the PChlides (although the intracellular PChlide level under light conditions is very low) is the main photoreceptor that in general antagonizes the UVB effect through the LHCII.

It is well established that UVB radiation induces the blockage of electron transport further than $Q_A^-$. Upon illumination with different wavelengths of ML, it can be observed that PChlides (620-640/442 nm) antagonize the inhibitory effect of UVB on the electron transport (PSIo) through LHCII.

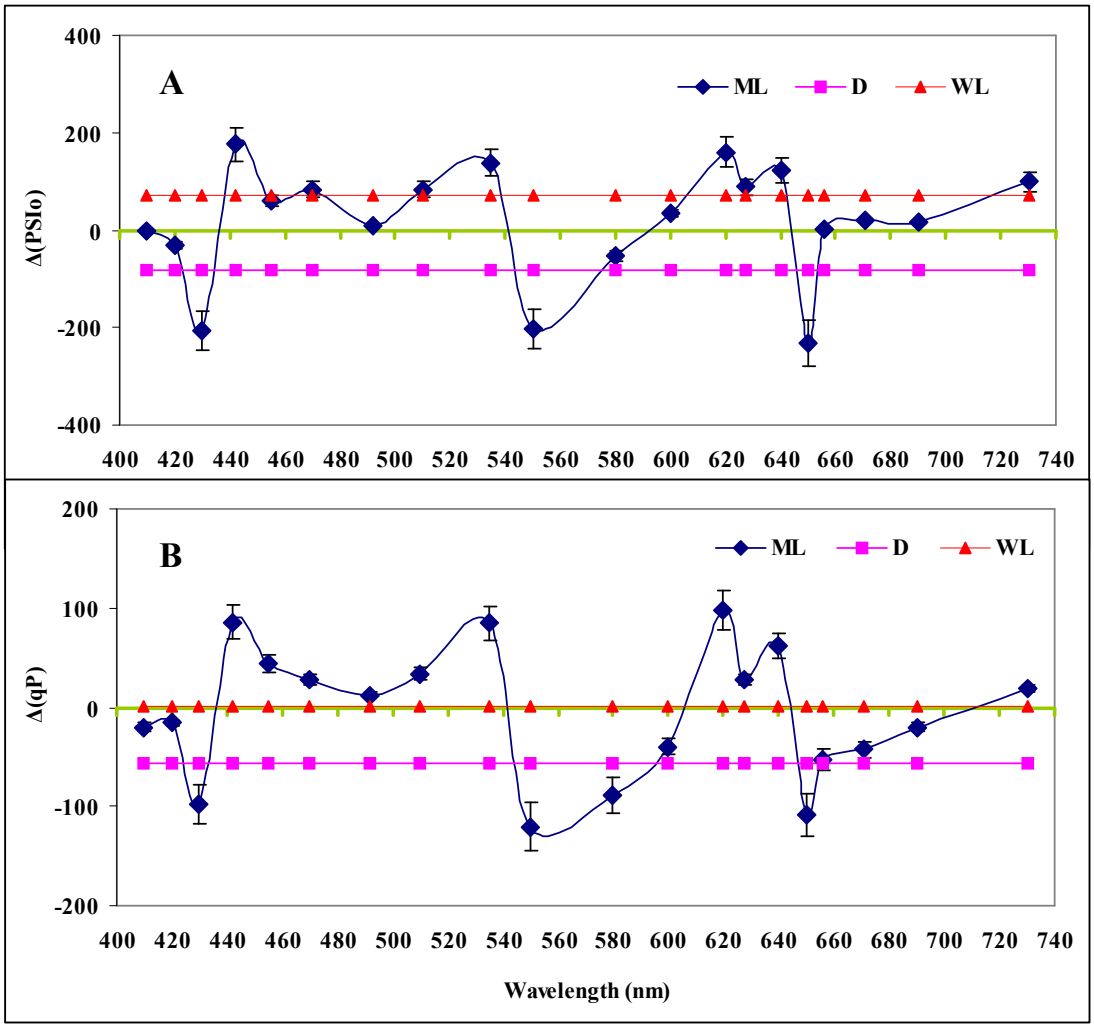


**FIGURE 19.** Action spectra of the (**A**) efficiency of electron transport further than $Q_A^-$ (ΔPSIo) and (**B**) photochemical quenching capacity (ΔqP). The green line represents the *wt-lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

In addition, an efficient photochemical quenching is developed by excitation of LHCII with far red light (730 nm) (Fig. 19B). In contrast, Chls (656/430 nm) enhance the inhibitory effect of UVB on the electron transport (Fig. 19A) and this leads to a decrease in the photochemical quenching (Fig. 19B).

Alterations in electron transport are responsible for the changes occurring in the excitation pressure developed by UVB radiation on the PSII (Fig. 20A). In addition to Chls, a photoreceptor (possibly an unknown carotenoid) that absorbs at 550 nm act synergistically with UVB radiation and leads to a 200% increase in the excitation

pressure. In contrast, the excitation of PChlides decreased the over-excitation exerted by UVB on the PSII functionality (Fig. 20A).

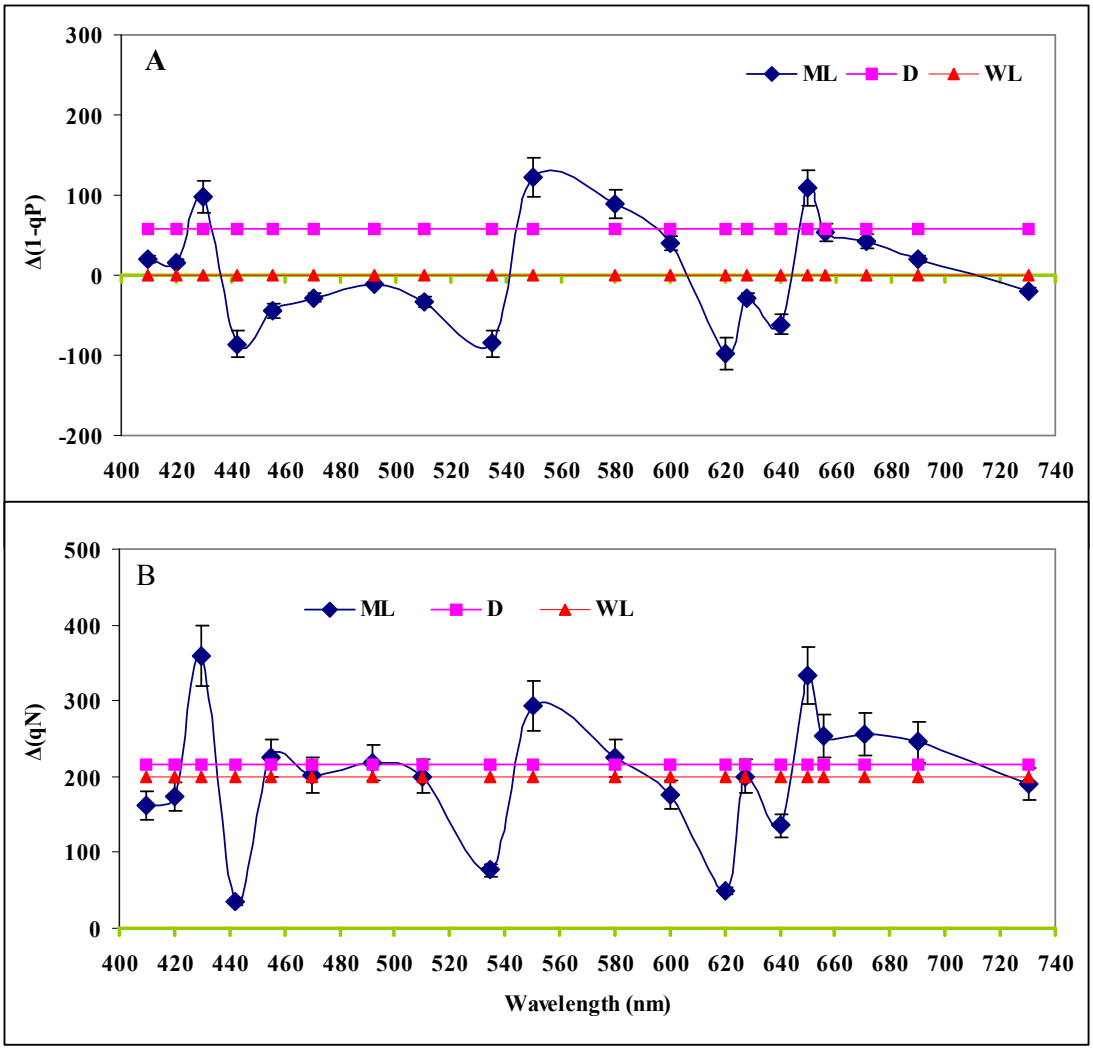


**FIGURE 20.** Action spectra of the (**A**) excitation pressure on PSII (Δ(1-qP)) and (**B**) non-photochemical quenching capacity (ΔqN). The green line represents the *wt-lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

By examining the action spectrum of non-photochemical quenching (qN), an enhancement of thermal dissipation of excess energy can be observed when the excitation pressure is high. Therefore the qN action spectrum is similar to the (1-qP) action spectrum (Fig. 20B). Previously, it was shown that the UVB treatment induces an increase in the amount of $Q_B$ non-reducing centers. The corresponding action spectrum shows clearly that Chls (656/430 nm) are the primary photoreceptors that enhance the

UVB effect by increasing the level of $Q_B$ non-reducing centers. PChlides (620-640/442 nm) also rises from the ranks as the parameter for the photoreceptor that regulates the attenuation of the UVB effect through the decrease of $Q_B$ non-reducing centers (Fig. 21). A similar effect was obtained for the excitation with far red (730 nm), suggesting the possibility that PSI is involved in the protection of reaction center functionality (Fig. 21).

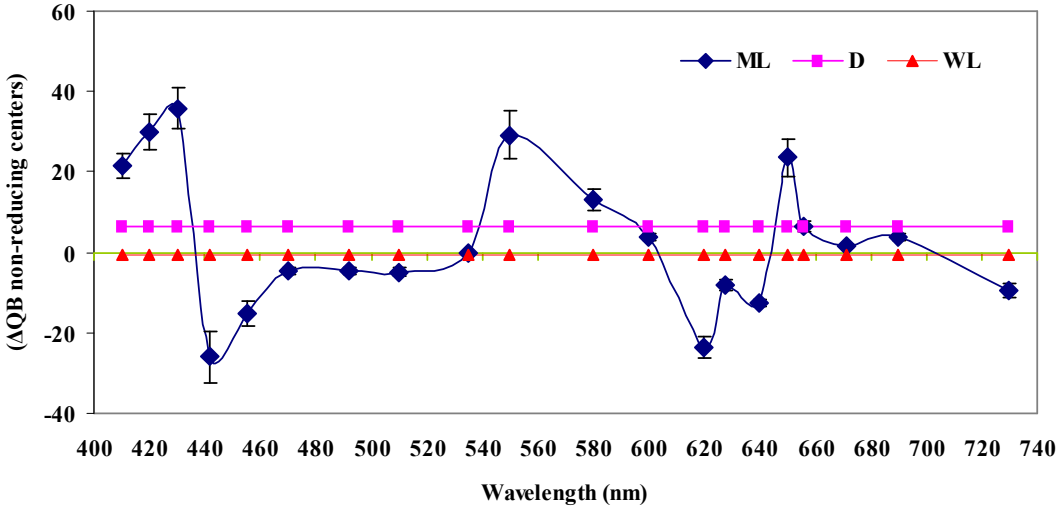


**FIGURE 21.** Action spectrum of the accumulation of $Q_B$ non-reducing centers. The green line represents the *wt-lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

Interesting alterations occur in the PSII heterogeneity (in *wt*+UVB cultures) upon excitation with monochromatic light. Although UVB radiation induced the decrease in quantity of the functional PSII-α reaction centers, there is a diminution of the UVB effect when LHCII is receiving light of 620-640 and 442 nm (absorption maxima of free and active PChlide). At these wavelengths the functionality of PSII-α reaction centers is increased approximately 30%. Similar effect to UVB radiation is obtained by excitation with 656 and 430 nm, which suggests the involvement of Chls in the amplification of UVB inhibitory effect on the functionality of PSII-α reaction centers. Evidently, the inactivation of PSII-α reaction centers occurs in parallel to the stimulation of the functionality of PSII-β and *vice versa* (Fig. 22).

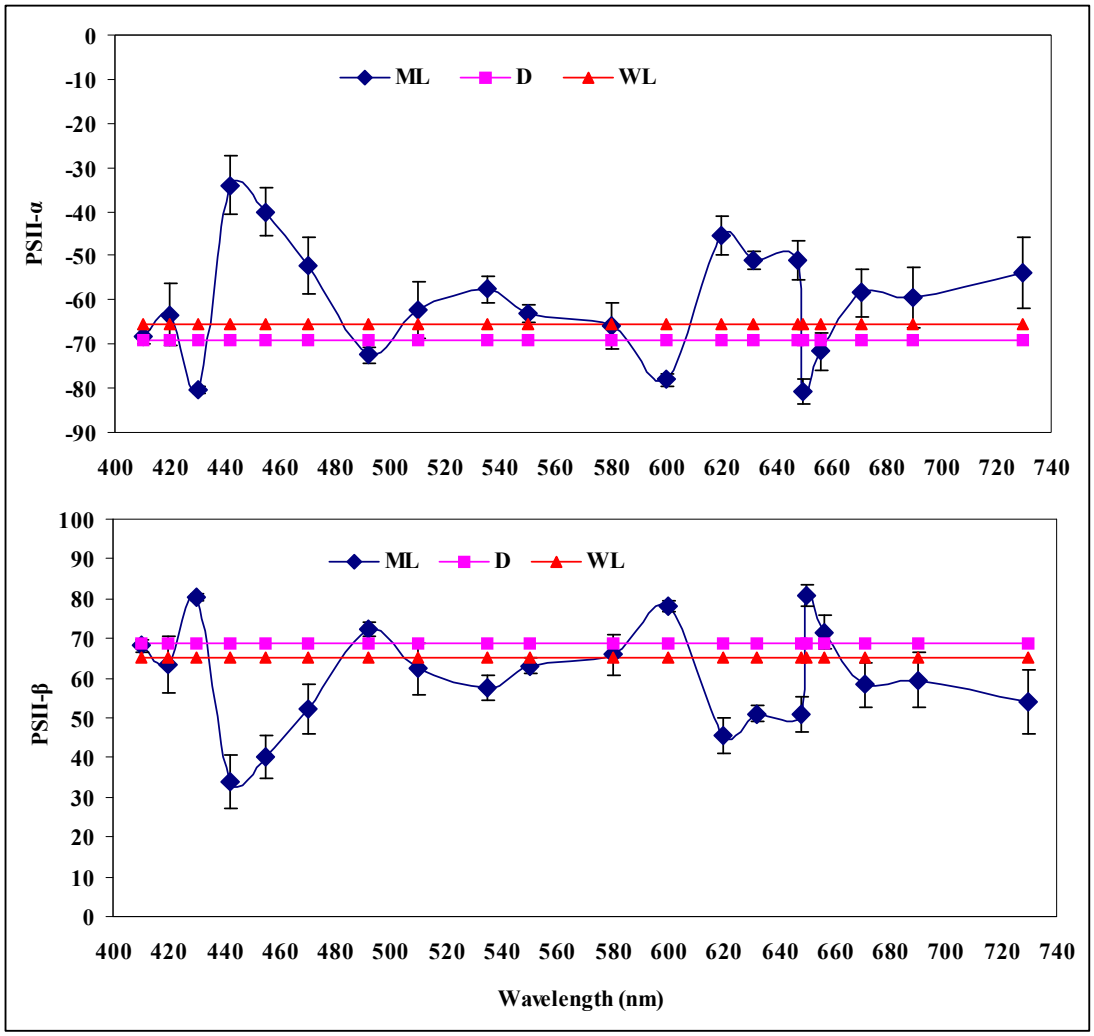


**FIGURE 22.** Action spectra of the functionality of PSII-α and PSII-β reaction centers in *wt* cultures upon irradiation with UVB in different background of ML, D or WL conditions. The values given on Y-axis are calculated after extraction of corresponding control values (as measured prior to UVB irradiation). Thus, at the value 0 on Y-axis is placed the corresponding control.

The changes occurring in the photochemical and non-photochemical quenching capacity put their signature on the operational quantum yield of PSII ($\Phi_{SPSII}$). As it was shown, UVB radiation induced the decrease of quantum yield efficiencies (Fv/Fm and $\Phi_{SPSII}$). LHCII is involved in modulation of the UVB effect, acting antagonistically or synergistically. Upon excitation of PChlides, there is a reduction of the UVB effect suggesting that this photoreceptor is acting antagonistically to UVB, by increasing the operational quantum yield of PSII (Fig. 23A).

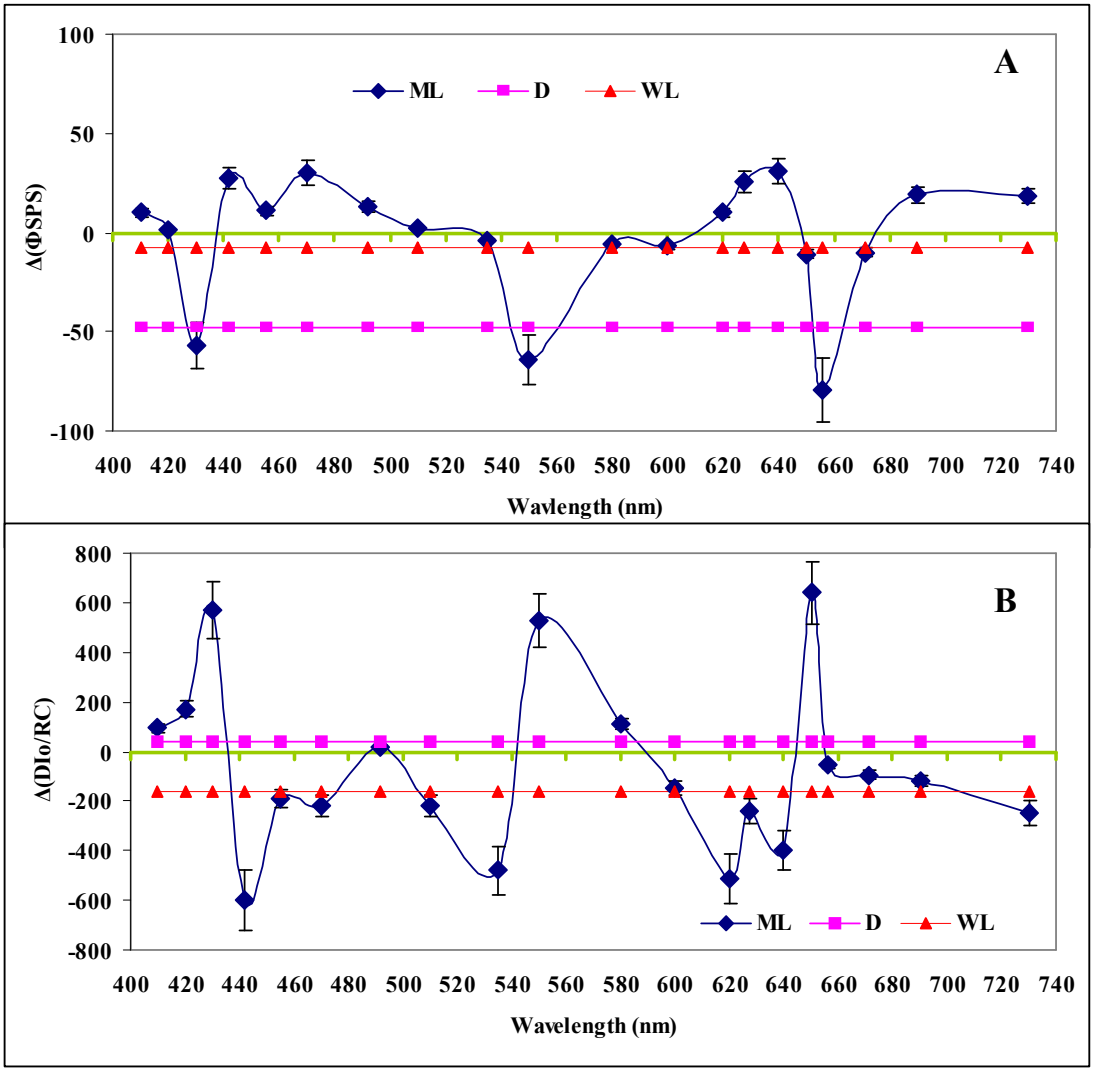


**FIGURE 23.** Action spectra of the (**A**) operational quantum yield of PSII ($\Delta\Phi_{SPSII}$) and (**B**) energy dissipated per reaction center ($\Delta$DIo/RC). The green line on X-axis, represents the *wt-lhc* (equal to 0), while ML, D and WL are the $\Delta$ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

In contrast, there is a strong decline in the $\Phi_{SPSII}$ upon irradiation with 656 and 430 nm, suggesting that Chls enhance the UVB effect. A clear positive influence in the reduction of UVB induced down-regulation of $\Phi_{SPSII}$ is also seen upon excitation with far red (730 nm) light, suggesting the involvement of PSI in the stimulation of LHCII efficiency (Fig. 23A). It seems that the reduction of UVB effect is associated with the increase in the photochemistry to the detriment of dissipation of excess energy (DIo/RC). At wavelengths where the PSII functionality is increased, there is a decrease in the amount of energy that is dissipated. Thus, the excitation of LHCII with certain

wavelengths, such as 620-640 and 442 nm, increases its effectiveness in the capture and transfer of light energy, by decreasing the energetic losses through LHCII. As result, the UVB effect is reduced and the photochemical reactions are enhanced. In opposition, the rate of energy dissipation is intense at 656 and 430 nm, resulting in decreased photochemical quenching and increased UVB effect by the synergistic participation of LHCII (Fig. 23B).

The overall result of the photoreceptors contribution to the regulation of UVB effect on the structure, conformation and function of the photosynthetic apparatus can be comprised in the photosynthetic performance index ($PI_{(abs)}$) (Fig. 24). The investigation of this parameter shows that the tolerance to UVB is increased upon irradiation with those wavelengths from the light spectrum that stimulate the effectiveness of the LHCII in the capture and energy transfer to the reaction centers.

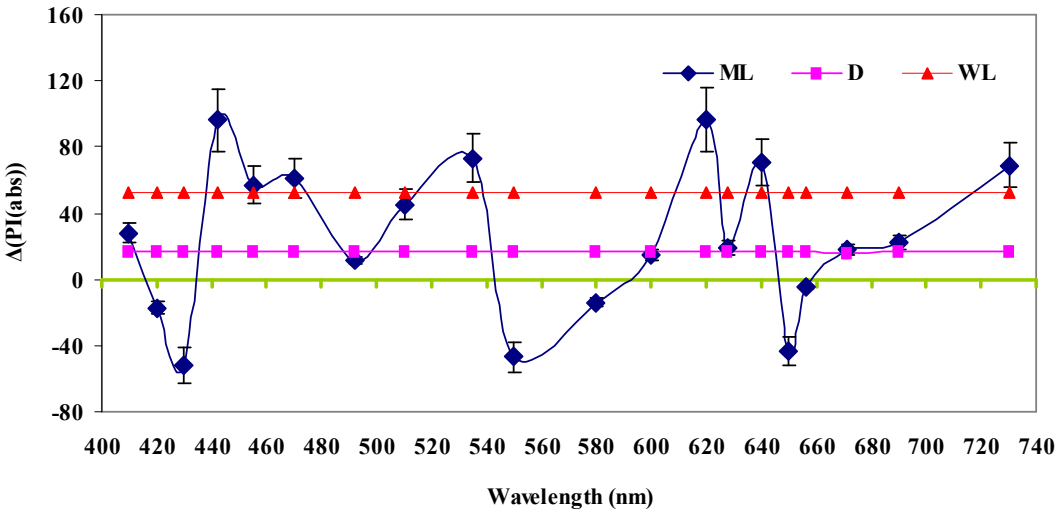


**FIGURE 24.** Action spectrum of LHCII contribution to the regulation of UVB effect on the performance index of the photosynthetic apparatus (ΔPI(abs)). The green line represents the *wt*-*lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

At these wavelengths, due to the intensification of photochemical quenching to the detriment of non-photochemical quenching, the UVB radiation effect is antagonized. This is true for the excitation with 442 and 620-640 nm which suggests the contribution of PChlides to increased effectiveness of the LHCII and subsequent tolerance of the photosynthetic apparatus to UVB. A similar effect seems to be exerted by the excitation of LHCII with 535 nm, as well as, by the PSI (at 730 nm). In contrast, the excitation of

Chls with 430 and 656 nm monochromatic light resulted in the intensification of UVB effect through LHCII (Fig. 24).

## 2.2. ACTION SPECTRA OF THE RECOVERY ABILITY OF THE PHOTOSYNTHETIC APPARATUS

The recovery ability of cultures irradiated with UVB under different wavelengths of ML is exhibited only by the *wt*-UVB(R) cultures. For the *wt-lhc*-UVB(R) mutant none of the UVB-irradiated cultures recovered, confirming the previous assertion that the LHCII is essential for restoration of photosynthetic apparatus functionality after UVB stress. The action spectrum of Δ(Fv/Fm) illustrated in Fig. 25 shows clearly that the same photoreceptors responsible for the tolerance of the photosynthetic apparatus against the UVB radiation (active and inactive PChlide, an unknown carotenoid absorbing at 535 nm and the reaction center of PSI) also seem to be responsible for the recovery of photosynthetic apparatus functionality after the cessation of UVB treatment.

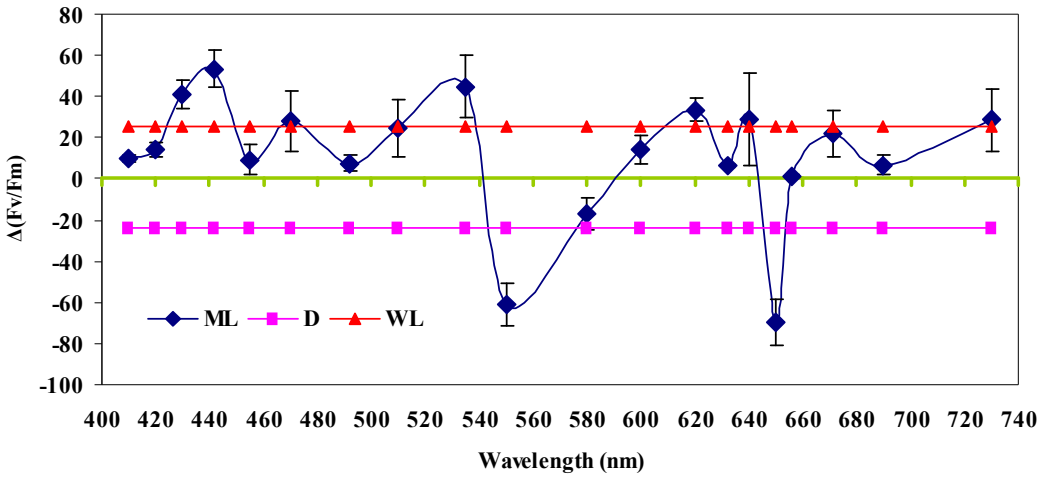


**FIGURE 25.** Action spectrum of the contribution of LHCII to the recovery of the photosynthetic apparatus functionality (described as ΔFv/Fm) from the UVB effects. The green line represents the *wt-lhc*, while ML, D and WL are the Δ values obtained for cultures exposed in monochromatic light of different wavelengths (nm), dark and white light conditions. For additional details, see legend Fig. 17.

## *DISCUSSION*

The present investigation characterizes the main effects of UVB on bioenergetics in the *wt* and in *wt-lhc* (mutant without LHCII) photosynthetic apparatus in order to understand the LHCII contribution to the behaviour adopted by the photosynthetic apparatus to UVB, as well as, the primary photoreceptors responsible for the regulation of photosynthetic apparatus sensitivity to UVB radiation and its recovery ability through changes in the LHCII.

1. The role of LHCII in the photosynthetic apparatus sensitivity/tolerance to UVB radiation

As it can be observed from the O-J-I-P transients (Fig. 1), both cultures (*wt* and the mutant *wt-lhc*) upon UVB treatment exhibited a similar pattern of changes, which express the inhibition of PSII functionality by UVB radiation. Specifically, the UVB treatment affected the I-step of the fluorescence rise, thus the typical O-J-I-P transient nearly became O-J-P transients after treatment. Both J-I and I-P transients were shown to disappear after the damage of OEC (Srivasatava et al., 1997). Thus, the large decline in the magnitude of the I-step caused by the exposure to UVB might indicate an impairment of PSII on the donor side (Fig. 1). In addition, UVB induced a higher $F_0$ fluorescence rise, especially in *wt*+UVB culture. The increase in $F_0$ fluorescence has been recognized as one of the most direct signs of photoinhibition (Aro et al., 1993). It was correlated with the occurrence of reaction centers with damage at the acceptor side of PSII (Vass et al., 1992). This resulted in a mismatch between light-driven electron transport and the capability of the Calvin cycle to accept electrons, which induces the over-reduction of the photosynthetic electron transport chain. This fact was also demonstrated by the data obtained from DCMU-poisoned samples (Fig. 2). The overall result is the transformation of a fraction of PSII centers into inactive reaction centers (Fig. 3). Inactivation of PSII primarily involves the specific degradation of the protein Dl located in photochemical reaction centre complex (Aro et al., 1993). The degradation of Dl elicited by either triplet P680 or singlet oxygen is usually greater in plants experiencing photoinhibitory damage. However, upon UVB irradiation, the turnover of the Dl protein is altered and the rate of degradation surpasses the rate of synthesis (Barbato et al., 2000). A study performed by Barbato et al. (1999) in barley *wt* and its chlorophyll b-less mutant *chlorine f2*, showed that UVB promotes the dephosphorylation of D1 and D2 reaction center proteins in parallel with their degradation. The loss of D1

and D2 proteins in the mutant was found to be slightly faster than in the *wt*, which is in agreement with the data presented here (Fig. 3). The inactivated reaction centers contribute to the increase in the functional antenna (ABS/RC) values (Fig. 4). Supplementary increase observed in *wt* is caused by the LHCII enlargement, as previously showed in Results and discussion-*Chapter I*. An increase in the functional antenna size results in a proportional enhancement of energy dissipation (Fig. 4) and of interest in an increase of energetic connectivity between photosynthetic units (Fig. 5).

Generally speaking, the UVB irradiation treatment reduced to a similar extent the photosynthetic rate (Fig. 9) and the maximal quantum efficiency of PSII (Fig. 7) in both *wt* and *wt-lhc* cultures. The LHCII seems to be responsible for the difference in the magnitude of changes regarding several parameters such as the quenching capacity (Figs. 4, 6C, D), the functional antenna size (Fig. 4) and the excitation pressure exerted by UVB on the PSII (Fig. 6A). In addition, in the presence of LHCII the photosynthetic units are connected in a higher degree than in the mutant *wt-lhc*+UVB culture. In the *wt* cultures not treated with UVB (i.e. the cultures used as control), the higher energetic connectivity existent between the photosynthetic units (expressed as pG) determines a better photosynthetic efficiency, although the density of active reaction centers is lower than in the mutant (Fig. 8). Under UVB stress conditions, two contradictory phenomena were observed in cultures possessing the LHCII antenna. First, a higher energetic connectivity (Fig. 5) is associated with an increase in the functional antenna size (Fig. 4) and an increased non-photochemical quenching capacity (Figs. 4, 6D). Moreover, all these changes were accompanied by the increase in the excitation pressure exerted on PSII by UVB radiation (Fig. 6A). Second, in spite of the first category of responses, the photosynthetic efficiency (Fig. 7) and the maximal net photosynthetic rate (Fig. 9) were almost similarly decreased in the *wt*+UVB culture as in the *wt-lhc*+UVB mutant. The existent contradiction between the two categories of responses could be formulated as the following question: why is the final result of UVB treatment similar in both cultures and conditions in which the higher excitation pressure exerted by UVB on PSII in the presence of LHCII (*wt* +UVB culture) should result in a higher rate of damage? Furthermore, another question arises: why does a photosynthetic apparatus possessing LHCII antenna the only type able to recover the damage induced by UVB?

To answer to these questions, one must take into account the fact that in *wt*+UVB culture the amount of light energy absorbed by a bigger antenna is higher than in a

mutant *wt-lhc* photosynthetic apparatus. Due to the blockage of electron transport to $Q_B$, there is an increase in the PSII over-excitation. In parallel, a bigger antenna contributes to a higher rate of excess energy dissipation that, even in high excitation pressure conditions in the *wt* +UVB culture, aided in the maintenance of PSII activity. Although it was postulated that a decrease in the primary photochemistry (Fig. 7) is associated with a decrease in the energetic connectivity (Strasser and Stirbet, 1998, 2001), the data presented here are not in agreement with the above mentioned. Specifically, the connectivity increases in parallel to functional antenna (Figs. 4-5).

It seems that LHCII contributes to this grouping of the photosynthetic units probably by structural readjustments that help to dissipate the excess excitation energy and to protect the reaction centers upon the UVB stress. Due to this conformation, the *wt* photosynthetic apparatus has the ability to restore its functionality after the cessation of UVB treatment. This is a very important finding of the present study. The fact that only in the presence of LHCII it is possible to recover the photosynthetic apparatus functionality suggests the following scenario of structural and functional readjustments of the photosynthetic apparatus during its exposure to UVB light. In a *wt* photosynthetic apparatus, incubated in normal light conditions, the photosynthetic units are highly connected by means of LHCII and this conformation assures a higher PSII efficiency so that there is no need to work numerous centers (Fig. 8). When the photosynthetic apparatus is exposed to UVB some reaction centers from the connected photosynthetic units are inactivated and transformed to dissipative sinks (Fig. 3). In their place other active photosynthetic units are connecting by their LHCII to this network. As long as UVB stress persists and the inactivation of reaction centers increases, other active photosynthetic units are added, to replace the inactivated ones. These units assure the photochemistry (Fig. 6B-C), whilst the inactivated ones contribute to the dissipation of excess energy and/or energy recycling (Fig. 4).

The data presented in Results and Discussion-*Chapter I* demonstrated that first candidates for the UVB-induced inactivation are the PSII-α reaction centers (RCs) which possess a bigger antenna than PSII-β RCs and are localized in grana, whilst the second type of PSII is presented in stromal thylakoids. Thus, the inactivated PSII α-reaction center network created at the beginning persists as long as the stressor and, subsequently, the PSII-α RCs become gradually inactivated (in *Chapter I* it was shown a strong correlation between the inactivated PSII-α fraction and $Q_B$ non-reducing centers). The

functionality of the system will be assured, in the last instance by PSII-β reaction centers. Since they are located in different regions as PSII-α, it can be supposed that structural rearrangements in thylakoid membranes also take place. This restructuring induced by UVB and assured by LHCII might be described as an "umbrella", constituted of the inactivated reaction centers. This kind of structure protects the functionality of active reaction centers and, maybe, permits the repair of damaged reaction centers. Conformational re-adjustments of PSII units within the thylakoid membranes were reported to take place upon UVB irradiation (Masi and Melis, 1992) and may be resulted from protein–protein and lipid-mediated interactions (Ivanov et al., 2006). The different structural and functional roles played by LCII antenna in the photosynthetic apparatus (i.e. participation of LHCII in stabilizing the granum ultrastructure and in the assembly of chirally organized macrodomains of PSII particles, which is thought to be responsible for the spatial separation of the two photosystem) (Horton et al., 1996, 2000; Chow et al., 2005) suggests that it can contribute to the formation of such dissipative structure upon light/UVB stress.

Another fact supporting the proposed model may be the finding that *in vitro* LHCII trimers form macroaggregates with a high potential for energy dissipation (Pascal et al., 2005). After the cessation of UVB treatment, as the damaged reaction centers are repaired they are disconnected from the "umbrella" and by their reconnection to other active photosynthetic units they reassure the initial structural and functional status. In contrast to these events occurring in *wt*, in the mutant *wt-lhc* photosynthetic apparatus, due to the lack of LHCII, such structural rearrangements are not possible and the reaction centers are gradually inactivated (Fig. 3) even after the cessation of UVB treatment. The results presented here can be interpreted as being in support of the hypothesis that photoinactivated PSII complexes photoprotect functional neighbors (Chow et al., 2005). Recent experimental data obtained by Lee et al. (2001) demonstrated that a photoinactivated PSII is initially only a weak quencher of excitation energy, but becomes a much stronger quencher during prolonged illumination when a substantial fraction of PSII complexes has also been inactivated. Thus, perhaps quenching by a photoinhibited reaction center is a useful response that provides photoprotection of thylakoid membranes (Chow et al., 2005). In addition to these data, the results presented herein demonstrate that the protective function of inactivated reaction centers is not possible without the participation of LHCII, which contribute to

structural readjustments of thylakoid membranes ("umbrella"). Therefore LHCII is the balance that counteracts the damage induced by UVB by increasing the dissipation of excess energy, whilst it is necessary for structural readjustments that avoid the irreversible destruction of the photosynthetic apparatus.

Important information about mechanisms contributing to the protection of the photosynthetic apparatus upon UVB irradiation was found after the investigation of carotenoid pattern. Chl and Car of green algae such as *Scenedesmus* sp. were found to play a structural and functional role in both photosystems (PSII and PSI) similar to those of higher plants (Senger et al., 1993; Bishop, 1996). Additionally, xanthophyll pigments are specifically distributed in pigment–protein complexes of photosynthetic membranes and these distributions are related to structural and photobiophysical functions of the pigments (Caffarri et al., 2001; Liu et al., 2004; Snyder et al., 2004).

The quantitative and qualitative analysis of carotenoids in *wt* and *wt-lhc* cultures shows that the amount of pigments is much lower in the mutant cultures than in the *wt* one (Fig. 13). The most abundant carotenoid is lutein, which is important in the development of PSII activity (Humbeck et al., 1989) and the reconstitution of LHCII (Plumley and Schmidt, 1987). Upon UVB irradiation, there is an increase in the total Car amount in the *wt*+UVB culture (Fig. 13). As it was demonstrated in the previous chapter this is probably related to the increase in the LHCII size. Concurrently there are changes in the carotenoid pattern characterized by an increase in the accumulation of β-carotene (Fig. 14), which it is known to help in photoprotection by quenching the triplet states of Chl (Formaggio and Bassi, 2001). The most abundant carotenoids upon UVB irradiation are lutein in the *wt*+UVB culture and α-carotene in the *wt-lhc*+UVB culture. Although UVB increased the β-xanthophyll pool, the violaxanthin cycle is not operational. This is in agreement with the conclusion emitted by Pfündel et al. (1992) that UVB causes the inhibition of violaxanthin cycle (Fig. 15B). In contrast, there is an activation of Lx cycle in *wt*+UVB culture (Fig. 15D), suggesting that UVB did not inactivate the Vx-deepoxidase as was previously reported by other investigators (Pfündel et al., 1992).

It is known that Vx, Ax and Lx are substrates for violaxanthin de-epoxidase (VDE) *in vitro* (Yamamoto and Higashi, 1978; Matsubara et al., 2003), but there were different affinities of enzyme reported for Vx, Ax or Lx (Verhoeven et al., 1999; Matsubara et al., 2003; Snyder et al., 2004). Notably, fluorescence excitation spectroscopic analysis showed that Lx bound to LHCII cannot transfer energy to Chl a

(Matsubara et al., 2004), a behavior that has previously been described for Vx bound to the peripheral site V1 of LHCII (Ruban et al., 1999; Caffarri et al., 2001). These data suggest that Lx may be equivalent to Vx (Matsubara et al., 2004). Interesting is the fact that in *wt*-*lhc*+UVB there is an increase in the pool of β-xanthophylls (Vx+Ax+Zx) (Fig. 15A), whereas in *wt*+UVB cultures there is an increase in the accumulation of α-xanthophylls (Lx+L)(Fig. 15C).

The preferential accumulation of α-xanthophylls instead of β-xanthophylls in the *wt*+UVB culture might be explained by the requirements in lutein of the *wt* photosynthetic apparatus for an increase in the LHCII size upon UVB radiation. This is based on experimental data obtained for *S. obliquus* by Bishop (1996) showing that lutein is specifically required for the formation of the oligomeric forms of the LHCII (Fig. 15D). Recently, it was found that lutein 'locks in' a primary mechanism of photoprotection during photoacclimation, converting efficient light-harvesting antennae of a shade plant into potential excitation dissipation centers. It is hypothesized that lutein occupies sites L2 and V1 in light-harvesting chlorophyll protein complexes of photosystem II, facilitating enhanced photoprotection through the superior singlet and/or triplet chlorophyll quenching capacity of lutein (Matsubara et al., 2004). In fact there is a higher dissipation of excess energy that in *wt*+UVB culture (Fig. 4) paralleled by an accumulation of lutein (Fig. 15C-D). This is in agreement with the experimental data reported by Niyogi et al. (1997) who found that in addition to the xanthophyll cycle pigments (Zx and Ax), α-carotene-derived xanthophylls such as lutein, which are structural components of the subunits of the light-harvesting complexes, contribute to the dissipation of excess absorbed light energy and the protection of plants from photo-oxidative damage. Significant was the fact that Lx/L cycle is reversible. After the cessation of UVB treatment, there is an increase in the accumulation of Lx to the detriment of L in the *wt*-UVB(R) culture (Fig. 15D). This constitutes evidence that reversible conversion of Lx to L may provide an effective mechanism for sustained photoprotection, since L is a better quencher of singlet and/or triplet chlorophyll than Vx when bound to the L1 or L2 site of LHCII (Formaggio et al., 2001).

The investigation of polyamines revealed that the absence of LHCII resulted in the low intracellular polyamine level (Fig. 10A). In the *wt* culture, the polyamine content is about 5 times higher than in the *wt*-*lhc* mutant and it is further increase by UVB radiation treatment (Fig. 10A). At thylakoid level, UVB induced an increase in the

polyamine amounts in both cultures (Fig. 12A) but their pattern is differently changed in *wt* as compared to *wt-lhc* culture. This fact demonstrates the relationship between thylakoid-associated polyamines and LHCII. In the *wt-lhc*+UVB culture, there is an accumulation of Put (Fig. 12C) which resulted in an increased Put/Spm ratio (Fig. 12B), whereas inverse changes were found in the *wt*+UVB culture (increased Spm and low Put/Spm ratio) (Fig. 12B, E). In the *wt*+UVB culture a low Put/Spm ratio was found to be related to an increase in the LHCII size. This was demonstrated by previous reports designating a low Put/Spm ratio as indicative of a bigger LHCII antenna (Kotzabasis et al., 1999; Sfichi et al., 2004; Sfakianakis et al., 2006). This is also stressed by the fact that in the *wt-lhc*+UVB culture the absence of LHCII gives a higher Put/Spm ratio (Fig. 12B). Thus, the regulatory role of thylakoid-associated polyamines in UVB stress conditions is expressed when the LHCII is present.

Extrapolating these data on the results obtained from the investigation of bioenergetics of both UVB-irradiated *wt* and *wt-lhc* photosynthetic apparatus, it seems that the efficient dissipation of excess excitation energy as well as the recovery of the photosynthetic apparatus functionality after UVB stress requires changes in the pattern of polyamine-associated to thylakoids. Specifically, an increase in the Spm-associated to thylakoids results in an increase in the LHCII size, followed by an increase in the functional antenna size and excess energy dissipation. Possibly, the changes occurring in the pattern of xanthophylls, as well as, the activation of the Lx cycle are part of the response chain signaled by the polyamines. In the last instance, it can be hypothesized that polyamines may constitute one or more of the primary mechanisms induced by UVB. To gain more insights about the regulation of the LHCII during exposure to UVB action spectra constructed for both wt and *wt-lhc* cultures.

2. <u>The photoreceptors that regulate the photosynthetic apparatus sensitivity/tolerance to UVB radiation</u>

Previosuly, it was shown (*see* also *Chapter I*) that the signal for the structural and functional adjustments of the photosynthetic apparatus upon UVB radiation is given by the excitation pressure exerted on PSII. In this context, the photoreceptors that influence the sensitivity to UVB are in fact those that modulate the excitation pressure of PSII. Consequently, they determine the antagonistic or synergistic contribution of antenna to the UVB effects by stimulating the photochemical or non-photochemical quenching

capacity of the photosynthetic apparatus, since both mechanisms are contributing to the reduction of the over-excitation of PSII.

From the action spectrum obtained for the excitation pressure of PSII (1-qP), one can see that excitation with ML of 442 nm and 620-640 nm decreased the excitation pressure exerted by UVB on PSII (Fig. 20A). At these light wavelengths, by increasing the photochemical quenching capacity in parallel to the diminution of thermal dissipation, there is an antagonistic action to the UVB effect (Figs. 19B, 20B). The photoreceptors sensitized by these wavelengths are the free protochlorophyllide (inactive PChlide absorbing in 620/442 nm) and the "active" Pchlide (absorbing in 640/442 nm). The inactive PChlide proves to be more efficient than the active one in preserving the reaction center functionality (Fig. 17B) and optimizing the photosynthetic performance (Fig. 17A). This is due to the contribution of PChlide to the reduction of the excitation pressure exerted by UVB on the PSII (Fig. 20A). Probably, inactive and active PChlides are involved in the capture of the excitation energy but they do not transfer this energy to chlorophylls of the LHCII (because they are not constituents of the LHCII) and therefore, they contribute to the minimization of the PSII over excitation.

The "active" PChlide (640/442 nm) appears to be more efficient than the inactive one to cope with the UVB effects on PSII functionality. This photoactive form, in addition to the above mentioned inactive one, can be photoconverted to chlorophyllide (Chlide) within a few seconds (Kotzabasis et al., 1990) and further, light independently, to chlorophyll. It is possible that at least a part of the excitation light energy absorbed by the active PChlide is udse for its conversion (PChlide to Chlide) and therefore, it contributes to the decreasing of PSII excitation pressure (Fig. 20A).

Additionally to PChlides, the action spectra revealed the possible involvement of an unknown carotenoid, which by excitation at 535 nm leads also to the reduction of the PSII excitation pressure (Fig. 20A), by contributing to an increase in the electron transport rate (probably due to the efficiency of light capture) and photochemical quenching capacity (Fig. 19A-B). By increasing the efficiency of energy transfer between the pigment molecules in the LHCII there is an increase in the amount of excitons that reaches the reaction centers, simulating a photoadaptation of the photosynthetic apparatus to HL conditions. This means the increase of the active reaction centers (Fig. 17B) and a decrease of the functional antenna-size (Fig. 18). As a consequence of this situation, there is an enhancement in the photochemistry of the

photosynthetic apparatus (Fig. 19B) and, therefore, a preference for the state 2 (i.e. LHCII-transfer to PSI (Haldrup et al., 2001). Under these conditions, the light absorbed by the PSI (about 700 nm) could strongly contribute to the additional protection of the PSII and enhancing the photochemical quenching (Fig. 20B), minimizing in parallel the excitation pressure of PSII (Fig. 20A). Indeed, all the action spectra demonstrated that longer wavelengths (>690 nm) contribute to an improvement of the photosynthetic performance of PSII (Figs. 17A, 23A, 24). All these photoreceptors [inactive and active PChlide, an unknown carotenoid and the PSI reaction center ($P_{700}$)] decrease the excitation pressure exerted on PSII by UVB by stimulating the photochemical quenching and the electron transport rate (Fig. 19). Thus, the effectiveness of these photoreceptors consists in the fact that they contribute to an increase in the functionality of reaction centers (Figs. 17B, 21) which assures higher photochemical quenching. In other words, they induce similar responses as that obtained for HL-adaptation, such as decreased antenna size, increased electron transport rate (Bailey et al., 2001), making the photosynthetic apparatus more tolerant to UVB radiation.

In opposition to the beneficial effects obtained by excitation with 620-640/442 nm, 535 nm and 690-730 nm, the functionality of the photosynthetic apparatus is negatively affected by UVB upon excitation mainly with 656/430 nm. At these wavelengths, the excitation pressure of PSII is significantly increased (Fig. 20A), probably due to electron transport inhibition (Fig. 19A) and a decreasing in the capacity of photochemical quenching (Fig. 19B). As result, there is a decrease in the density of active reaction centers (Figs. 17B, 21) which leads to a higher functional antenna size (Fig. 18). In addition, the amount of functional PSII-α reaction centers decreased on the favor of PSII-β reaction centers (Fig. 22). The negative peaks obtained at 656/430 nm suggests that chlorophylls (Chl a and b) are involved in the intensification of UVB effects in the photosynthetic apparatus, as a result of amplification of the excitation pressure exerted on PSII (it is known that Chl is the main pigments of the LHCII). The overall result is the reduction in the efficiency of quantum yields (Figs. 17A, 23A) and photosynthetic performance of the PSII (Fig. 24) accompanied by an increase of energy dissipation by non-photochemical quenching (Figs. 20B, 23B). Thus, at the respective wavelengths the obtained responses are similar to those induced by UVB irradiation under low light conditions (*see* Results and Discussion-*Chapter I*) and the sensitivity of the photosynthetic apparatus to UVB is increased.

The data presented herein show that some photoreceptors are responsible for the antagonistic effect to UVB and increase the tolerance of the photosynthetic apparatus to UVB, whereas others have a synergistic effect to UVB radiation that results in increased sensitivity of the photosynthetic apparatus to UVB. In this context, the question which arises is related to the role of these photoreceptors in the modulation of the polyamine-associated pattern which seems to be the primary response to UVB radiation (*see* Chapter I). De facto, UVB induces the increase in the Spm associated to thylakoids (and/or decrease of Put level) which leads to an increase in the size of antenna and NPQ capacity. Unfortunately, data is scarce regarding the regulation of polyamine levels by photoreceptors. Previous reports of Kotzabasis et al. (1999) showed that the active PChlide acts as photoreceptor of the decrease in the level of the Put/Spm ratio and this is in agreement with the results of the present contribution. It seems that the other mentioned photoreceptors (Chls) enhance the over-excitation exerted by UVB on PSII and this increases the thylakoid-associated Spm (decrease of the Put/Spm ratio) that simulates a LL-adapted photosynthetic apparatus (with a similar phenotype with the UVB induced one), therefore, resulting in the amplification of the UVB effect (increasing the sensitivity to UVB). In contrast, the excitation of another series of primary photoreceptors (Pchlide, PSI reaction center and unknown carotenoid) increases the thylakoid associated Put (increase of Put/Spm ratio) simulating a HL-adapted photosynthetic apparatus with an increased photochemical quenching capacity. As a result, the decrease of the PSII excitation pressure acts antagonistically to the UVB effect and this leads to an enhanced tolerance of the photosynthetic apparatus against UVB. In addition to and in agreement with this data it was demonstrated that Put also stimulates the chemiosmotic APT synthesis on the thylakoids (more than 70%) without any changes in the light conditions (Ioannidis et al., 2006), inducing the photochemical quenching. The restoration of the PSII functionality after irradiation with UVB in ML conditions is possible only in *wt* cultures. This confirmed the previous assertion that LHCII is essential for the restoration of photosynthetic apparatus functionality after UVB stress. The action spectrum of ΔFv/Fm (= Fv/Fm(*wt*) – Fv/Fm(*wt-lhc*) shows that the same photoreceptors responsible for the tolerance of the photosynthetic apparatus against the UVB radiation, as expected, also seem to be responsible for the recovery of the photosynthetic apparatus after the cessation of the UVB radiation (Fig. 25).

## *CONCLUSIONS*

Based on the preceding data, the main conclusions that can be drawn here are the following:

1. LHCII and inactivated reaction centers contribute to the building of dissipative units that assure protection to the remaining active reaction centers and recovery of the photosynthetic apparatus.

2. Polyamine-associated to thylakoids (Put and Spm) regulate the conformational status adopted by the photosynthetic apparatus during UVB and, subsequently, its functionality, by adjusting the size of LHCII; in the absence of LHCII antenna, polyamines do not seem to be involved in the regulation of the photosynthetic apparatus response to UVB.

3. A higher excitation pressure is compensated by an efficient system of photochemical/non-photochemical energy quenching that is assured in the presence of LHCII.

4. A photosynthetic apparatus without LHCII has no potential to recover the damage induced by UVB. In the LHCII absence (*wt-lhc* mutant) the photosynthetic units work independently and do not protect each other.

5. The reduction of the PQ pool seems to be specifically UVB regulated but it has different affects on the culture responses by means of LHCII.

6. Series of action spectra and the difference of action spectra between *wt* and *wt-lhc* cultures (Δ(*wt* – *wt-lhc*)) show clearly that three primary photoreceptors (active and inactive PChlide (620-640/442 nm), an unknown carotenoid absorbing at 535nm and the reaction center of PSI (690-730 nm) increase the tolerance of the photosynthetic apparatus to UVB by decreasing the excitation pressure exerted on PSII by UVB. Their stimulation induced responses similar to HL adaptation (the functional antenna size is kept low, the density of active reaction centers increased, the amount of $Q_B$ non-reducing centers decreased the

functionality of the photosynthetic apparatus is assured by PSII-α reaction centers and all these increase the photosynthetic efficiency). The same photoreceptors are responsible for the recovery of the photosynthetic apparatus after the cessation of the UVB radiation.

7. Chlorophylls (Chl a and b) are the primary photoreceptors responsible for the enhanced sensitivity of the photosynthetic apparatus against UVB radiation causing an increase in the excitation pressure exerted on PSII by inducing an increase in non-photochemical quenching (NPQ) accompanied by a series of responses that simulate LL-photoadaptation (the functional antenna size increased, the density of active reaction centers decreased, the amount of $Q_B$ non-reducing centers increased and all these decrease the photosynthetic efficiency).

Since polyamines regulate the size of LHCII, which determines the overall behavior adopted upon UVB it can be supposed that they act beyond LHCII. It is not clear if their pattern is PQ-regulated or there are other mechanisms acting beyond PQ redox state changes that make polyamine to act as regulators of LHCII and consequently, of the photosynthetic apparatus sensitivity to UVB. This ultimately means that polyamines can constitute one among many or even the primary site of UVB action.

Thus, corroborating the data obtained in *Chapter I* and *Chapter II* it becomes clear that the LHCII plays a key role in the determination of the degree of sensitivity to UVB radiation. Since the above results suggest that polyamines can be involved in the regulation of LHCII, the next step was to investigate the involvement of polyamines, mainly of those associated to thylakoids, in the sensitivity of the photosynthetic apparatus to UVB, with a focus on their effect on the antenna size. This was done by altering the titer of Put and Spm in thylakoids and investigating how this change impacted on the photosynthetic behavior exhibited to UVB in the presence or absence of LHCII.

# *CHAPTER III*

## POLYAMINE - REGULATED MECHANISM OF THE PHOTOSYNTHETIC APPARATUS SENSITIVITY/TOLERANCE TO UVB RADIATION

## *RESULTS*

The previous data (*see* Results and Discussion-*Chapter 1*) demonstrated that the behavior expressed by the photosynthetic apparatus to UVB irradiation mimics low light photoadaptation. This can be described as a pattern of changes originating from the LHCII size increase and inactivation of PSII reaction centers and finalized with the reduction in photosynthetic capacity. The importance of PAR intensity in the modulation of behavior exhibited in the presence of UVB radiation resides in the diminution or enhancement of the UVB effect on antenna size. Low PAR intensities increase the UVB effect by inducing a bigger antenna size and, therefore, the UVB-induced inhibition of the photosynthetic capacity becomes more pronounced in LL conditions. In contrast, high PAR intensities act antagonistically to UVB radiation. The inhibitory effect of UVB on the photosynthetic capacity is diminished by reducing the LHCII antenna size. In addition, it was discovered that the alterations in LHCII size are subsequent to changes in the pattern of thylakoid-associated polyamines, specifically in the Put/Spm ratio. A lowering of the Put/Spm ratio accompanies an increase in the LHCII size, whereas an increase of the Put/Spm ratio leads to the LHCII size reduction. Thus, these data suggest a possible regulatory role for Put/Spm ratio on the LHCII size. Furthermore, the fact that only a *wt* photosynthetic apparatus has the ability to recover from changes induced at the structural and functional level by UVB (*see* Results and Discussion-*Chapter II*) indicates that the LHCII is absolutely necessary for the restoration of the photosynthetic activity after UVB exposure.

Corroborating the data obtained in *Chapter I* and *Chapter II* it appears that the photosynthetic apparatus sensitivity to UVB and its recovery capacity depend on the LHCII size and polyamines might play a regulatory role. Consequently, it was necessary to delve more deeply into the interrelationship of these two factors, LHCII size and thylakoidal Put/Spm ratio. To achieve this, the effect of UVB was studied in cultures incubated with exogenously added polyamines. The rationale used in designing the experiment was simple and based on the above observations in addition to data reported

by Kotzabasis et al. (1999) who found that alterations in the Put/Spm ratio induce alterations in the antenna size. Specifically, if a decrease of Put/Spm ratio produces an increase in the LHCII size and, subsequently, an increase in the sensitivity degree to UVB, then an increase of Put/Spm ratio should be followed by a decrease of LHCII size and, subsequently, by an increase in the photosynthetic apparatus tolerance to UVB. Consequently, the *wt* cultures incubated in LL conditions (where the UVB effect was stronger than in HL conditions) were supplemented with 1 mM Put and exposed to UVB. On the contrary, if the decrease of Put/Spm ratio is responsible for the increase in antenna size and through this the photosynthetic apparatus sensitivity to UVB, then the addition of Spm in HL conditions (which antagonizes the UVB effect) should lead to the accentuation of sensitivity to UVB. As result, HL-incubated cultures were supplemented with 1mM Spm. The polyamine concentration used in the above experiments was established in function of data previously reported (Beigbeder et al., 1995; Navakoudis et al., 2001). Furthermore, additional experiments with exogenously added polyamines were made in *wt-lhc* cultures. Bioenergetic and molecular structure changes in the photosynthetic apparatus as result of polyamine titer alteration were investigated during 3 h of UVB exposure and additional 4h of incubation in conditions without UVB (*see* Material and Methods 2.3.3.).

## 1. POLYAMINE-REGULATED MECHANISM OF THE *wt*-PHOTOSYNTHETIC APPARATUS SENSITIVITY TO UVB

Under UVB irradiation, the supplementation of LL-incubated cultures with Put and of HL-incubated cultures with Spm resulted in a pattern of changes in the structure, conformation and function of the *wt*-photosynthetic apparatus that can be comprised in the following statement: Put+UVB induced a HL-simulated photosynthetic behavior that increases the tolerance to UVB, whilst Spm+UVB induced a LL-simulated photosynthetic behavior that is more sensitive to UVB. The evidence supporting this finding is described below.

### 1.1. POLYAMINE SIGNATURE ON THE PHOTOSYNTHETIC APPARATUS BIOENERGETICS

#### 1.1.1. Changes in the Chl *a* fluorescence polyphasic kinetics

The investigation of O-J-I-P transients in the cultures treated with polyamines as compared to the untreated control cultures reveals a significant polyamine-induced effect

on the both photochemical (O-J) and thermal (J-I-P) phases of the fluorescence rise. As depicted in Fig. 1, the culture treated with Put (LL+Put+UVB) as compared to the culture incubated in LL+UVB condition exhibited a faster and a higher O-J rise (the first 2 ms) that is followed by a slower rise until the I-step (30 ms) and then rises to the P-peak (about 150ms).

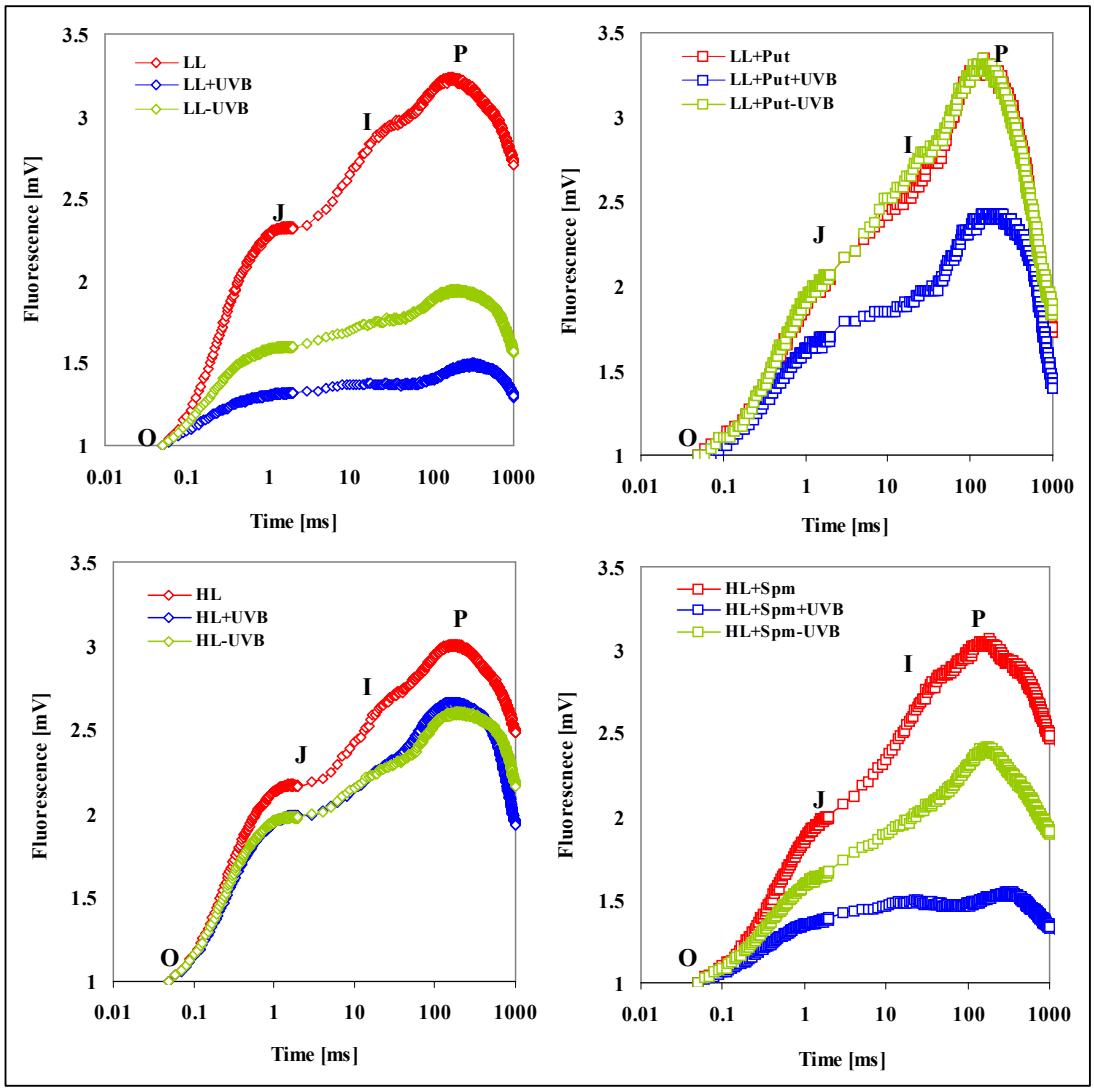


**FIGURE 1.** Changes in the polyphasic kinetics of Chl a fluorescence in cultures exposed to LL (**LL**) and HL (**HL**) upon addition of 1mM Put (**LL+Put**) or 1mM Spm (**HL+Spm**). The kinetics were registered from the data collected by Chl a fluorescence measurements at three experimental steps: prior to UVB irradiation treatment (*red curve*), after 3h of UVB irradiation treatment (*blue curve* "UVB"), and after an additional 4h of recovery in the initial conditions without UV-B (*green curve* "-UVB").

Put diminishes the suppression effect of UVB on the J-I fluorescence rise and this results in a higher Fm value. Besides the 50% reduction of the UVB quenching effect on Fm, Put assures a fast and complete recovery of fluorescence kinetics from the UVB effect. From all the experimental variants used, the restoration of Fm, in the presence of Put, is total. The overall picture of Put action in LL-incubated culture, during exposure to UVB radiation, resembles that obtained for HL-culture irradiated with UVB. In other words, Put is responsible for inducing a HL-effect in the LL-culture exposed to UVB, which antagonizes the serious consequences of UVB radiation treatment on the PSII functionality.

Contrary to Put, Spm induces an increase in $F_0$ value in the HL+UVB culture, which may express damage to PSII-oxidizing site (data not shown). Upon Spm addition, the J-P phase is strongly suppressed and the quenching of Fm is about 60% higher as compared to the values obtained for the HL+UVB culture (Fig. 1). On the whole, the quenching effect of Spm on the Chl a fluorescence rise indicates that PSII activity is highly impaired. The PSII functionality is partially recovered after the cessation of UVB effect, showing that the damage induced by UVB in the presence of Spm (in HL+Spm+UVB culture) is not irreversible. The general view of Spm action in HL-culture exposed to UVB is similar to that obtained for LL+UVB cultures. This means that Spm amplifies the UVB effect on the photosynthetic apparatus exposed to HL by inducing a LL-simulated behaviour.

### 1.1.2. The influence of polyamines on the alterations induced by UVB in the PSII photochemistry

There have been a large number of investigations carried out on UVB-stressed plants showing a decrease in the rate of oxygen evolution, electron transport and the ratio Fv/Fm (*see* Tevini, 2004 and references therein). The expression Fv/Fm is an excellent measure of the maximum quantum yield efficiency of PSII (Srivastava et al., 1997). The measurements shown in Fig. 1 indicate, as mentioned earlier that by exposure to UVB, the immediate effect is the decrease of Fm and a more or less pronounced increase of $F_0$ resulting in reduced variable fluorescence (Fv) and a decreased Fv/Fm ratio. Similar results have been published by other investigators (Krause et al., 1999; Heraud and Beardall, 2001). Recently, it was suggested that the decrease of Fv/Fm may be due to several effects such as: a decrease of the rate of primary charge separation; reduction in

the stabilization of charge separation; an increase in the recombination of the rate constant of the radical pair of the reaction centers, as well as the disconnection of some minor antenna from the PSII reaction center (Briantais et al., 1996).

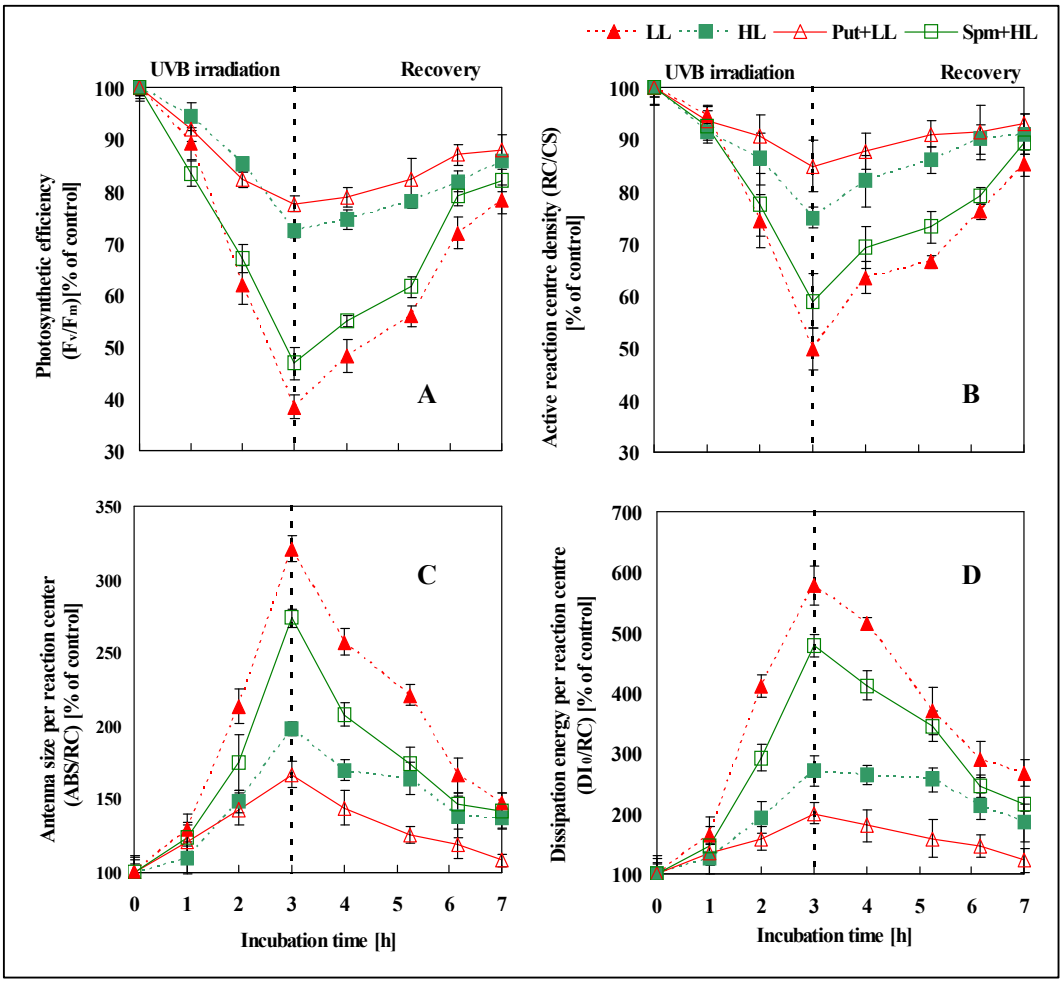


**FIGURE 2.** Kinetics of the (**A**) photosynthetic efficiency ($F_v/F_m$), (**B**) reaction center density (RC/CS), (**C**) functional antenna size (ABS/RC) and (**D**) rate of dissipation energy per reaction center ($DI_0/RC$), in the cultures incubated in different conditions of illumination during irradiation with 0.42 mW $cm^{-2}$ UVB and recovery. The values represent the means ± SD of three independent experiments. *Additionally*: LL, cultures incubated in low PAR intensity (87 µmol $m^{-2}$ $s^{-1}$) conditions; LL+Put, cultures treated with 1mM Put and incubated in LL conditions; HL, cultures incubated in high PAR intensity (650 µmol $m^{-2}$ $s^{-1}$) conditions; HL+Spm, cultures treated with 1mM Spm and incubated in HL conditions.

In Fig. 2 are shown the kinetics of several biophysical expressions, used to characterize the photosynthetic apparatus status such as Fv/Fm, RC/CS (the density of active reaction centers per cross section), ABS/RC (the absorbance per reaction centers that is a measure of the functional antenna size) and $DI_0/RC$ (the amount of energy

dissipated per active reaction center) in cultures supplied or not with polyamines during UVB irradiation and recovery. The kinetics recorded for Fv/Fm showed that the maximum quantum yield of PSII is strongly influenced by polyamines. Comparative to LL-incubated cultures (LL+UVB), where there is a Fv/Fm reduction of 60%, Put reduces the UVB effect by 40% and, therefore, the Fv/Fm in LL+Put+UVB culture is only 20% below the corresponding LL+Put control culture. On the contrary, the smaller effect of UVB on the Fv/Fm ratio in HL+UVB culture became more accentuated after Spm supplementation. In HL+Spm+UVB condition, the Fv/Fm ratio decreases from 25% (in HL+UVB) to 55% of the corresponding control values (Fig. 2A). Although the polyamine effect is evident during UVB radiation, after the cessation of UVB treatment there is no significant difference between the recovery ability of the cultures treated with polyamines as compared to the untreated cultures (Fig. 2A).

Examining the kinetics of functional antenna size (as expressed by ABS/RC), one can see that the increase in the Fv/Fm ratio in LL+Put+UVB culture is associated with a 150% reduction in the functional antenna size, as compared to the correspondent LL+UVB-culture (Fig. 2C). As a result of the inhibitory effect of Put on increasing antenna size by UVB irradiation, the $DI_0$/RC exhibits a similar decrease (Fig. 2D). At the same time, the density of active reaction centers increases to 40% (in LL+Put+UVB culture), as compared to the value obtained for LL+UVB culture (Fig. 2B). Overall, Put plays the same antagonistic effect to UVB radiation as HL intensities. It can be concluded that Put induces a HL-simulated photosynthetic apparatus that is able to counteract the negative effects of a mixture of LL and UVB radiation, by inhibition of antenna size enlargement as a response to UVB treatment. This leads to an increased tolerance of the photosynthetic apparatus to UVB radiation. In contrast to Put, Spm potentiates the UVB effect leading to changes that make a photosynthetic apparatus incubated in HL conditions have similar behavior as one exposed to LL. Specifically, in HL+Spm+UVB culture the functional antenna size increases strongly (Fig. 2C) in parallel with a reduction of the active reaction centers density (Fig. 2D). Consequently, the dissipation of light energy that cannot be used in photosynthesis (due to inactivation of reaction centers) increases about 100% (Fig. 2D) and the overall result is a reduction of photosynthetic efficiency (Fig. 2A). All these changes are consequences of the Spm effect on the antenna accentuating the photosynthetic apparatus sensitivity to UVB.

Independent to the experimental conditions applied, the UVB irradiated cultures express an increased potential to recover from the UVB effects (Fig. 2).

Previously, it was shown that LHCII participates in the energetic connectivity (expressed as pG) between the photosynthetic units in a structure ("umbrella") which dissipates the excess energy and protects the active reaction centers (*see* Chapter II). Thus, it is of interest to see if polyamines influence this characteristic of the photosynthetic apparatus during UVB stress conditions. As Fig. 3 shown, Put decreases the connectivity of photosynthetic units in the LL+Put+UVB culture about 200% as compared to the LL+UVB culture, an effect similar to what was found for the functional antenna size (Fig. 2C). In contrast, Spm increases the connectivity of the photosynthetic units in the HL+Spm+UVB culture and the magnitude of this response to UVB radiation can be compared to that found in the LL+UVB culture (Fig. 3A).

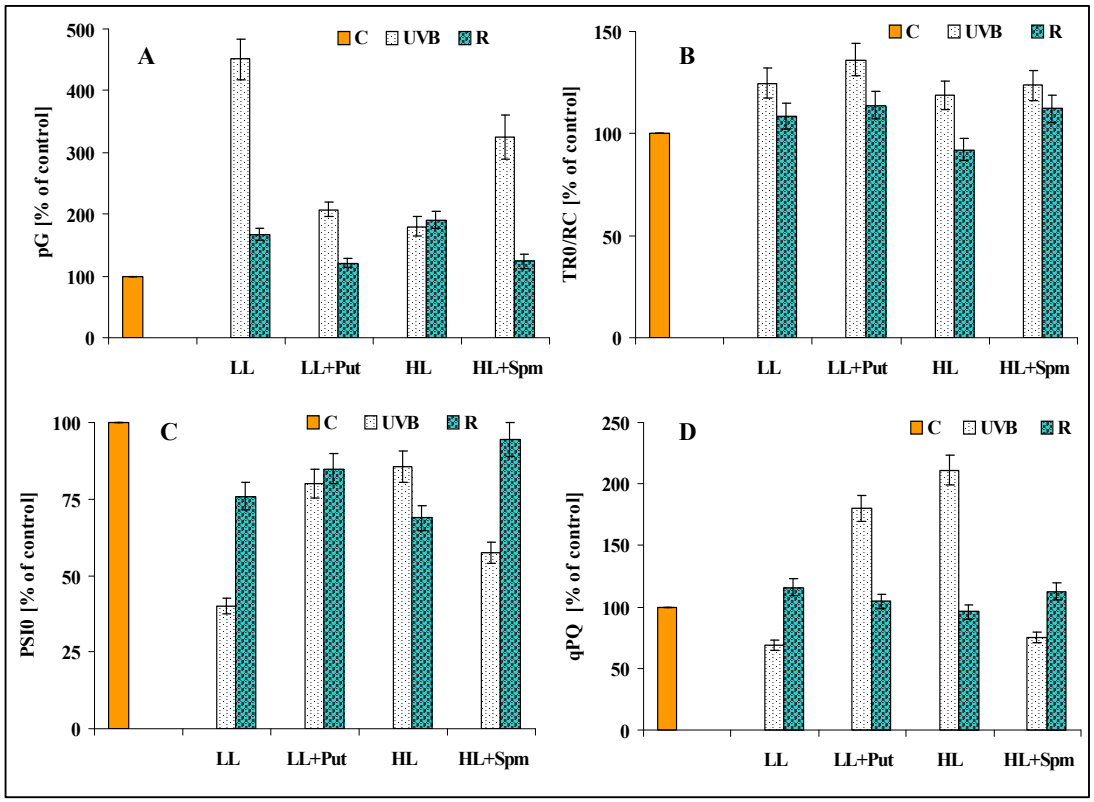


**FIGURE 3.** Polyamine effects on the (**A**) energetic connectivity between the photosynthetic units (pG), (**B**) efficiency of exciton trapping per reaction centers ($TR_0$/RC), (**C**) maximal rate of primary photochemistry ((PSIo) and (**D**) plastoquinone pool size (qPQ) after UVB irradiation (UVB) and recovery (R). The values are expressed as percentage from the corresponding control culture. For details see legend Figure 2.

UVB radiation treatment also increased the efficiency by which an exciton is trapped by the reaction center, leading to the reduction of $Q_A$ (described as $TR_0/RC$). In Figure 3B it is shown that $TR_0/RC$ is increased more by Put (in LL+Put+UVB culture), as compared to the correspondent LL+UVB culture, whereas it is not significantly affected by Spm (in HL+Spm+UVB culture). In spite of the fact that there are not significant differences between polyamine-treated and untreated cultures regarding the efficiency of $Q_A$ reduction upon UVB treatment, the electron transport (PSIo) shows changes that are the consequences of polyamine addition. Electron transport decreases under UVB radiation, especially in the LL+UVB culture as compared to the HL+UVB culture. The addition of Put inhibits the UVB-induced decrease in PSIo, as it is found in the LL+UVB culture. On the contrary, Spm amplifies the inhibitory effect of UVB radiation on the electron (Fig. 3C).

The alterations induced by UVB in PSIo are followed by similar reductions in the pool of oxidized PQ (described as qPQ) in cultures incubated in LL+UVB conditions. Significantly, Put inhibits completely the UVB effect in LL conditions (in LL+Put+UVB culture) and determines an increase (about 130%) in the oxidized PQ. By its action, Put induces a response that is similar to that observed in HL+UVB culture. In contrast, Spm cancels the positive effect of HL, which assures the maintenance of the PQ pool in an oxidized state during UVB treatment. As a result of Spm, qPQ is decreased (about 125%) in the HL+Spm+UVB culture and this response is similar to that found in LL+UVB conditions (Fig. 3D). All of the above changes induced by UVB radiation are reverted after the cessation of the UVB treatment. The capacity of cultures incubated in different experimental conditions to restore themselves to the initial status that existed before UVB is, roughly speaking, similar and not influenced by light conditions or polyamine treatments (Fig. 3).

### 1.1.3 Polyamine effects on the photochemical and non-photochemical quenching of the photosynthetic apparatus exposed to UVB in LL and HL conditions

The investigation of quenching properties of the photosynthetic apparatus exposed to UVB is important to understand the correlation between the dark and light reactions of photosynthesis and to assess through this the functionality of PSII. The parameters that are most representative and therefore used in quenching analysis are: the

excitation pressure exerted on PSII (1-qP), the non-photochemical quenching (qN) and the operational quantum yield of PSII ($\Phi_{SPSII}$).

Due to the reduction in the ability of algal cells to photosynthetically convert the absorbed light, the excitation pressure exerted on PSII increases upon UVB radiation. Excitation pressure is high in cultures incubated in LL condition (LL+UVB) but is kept low in HL+UVB culture due to the antagonistic protective effect of HL intensities (*see* Results and Discussion-*Chapter II*).

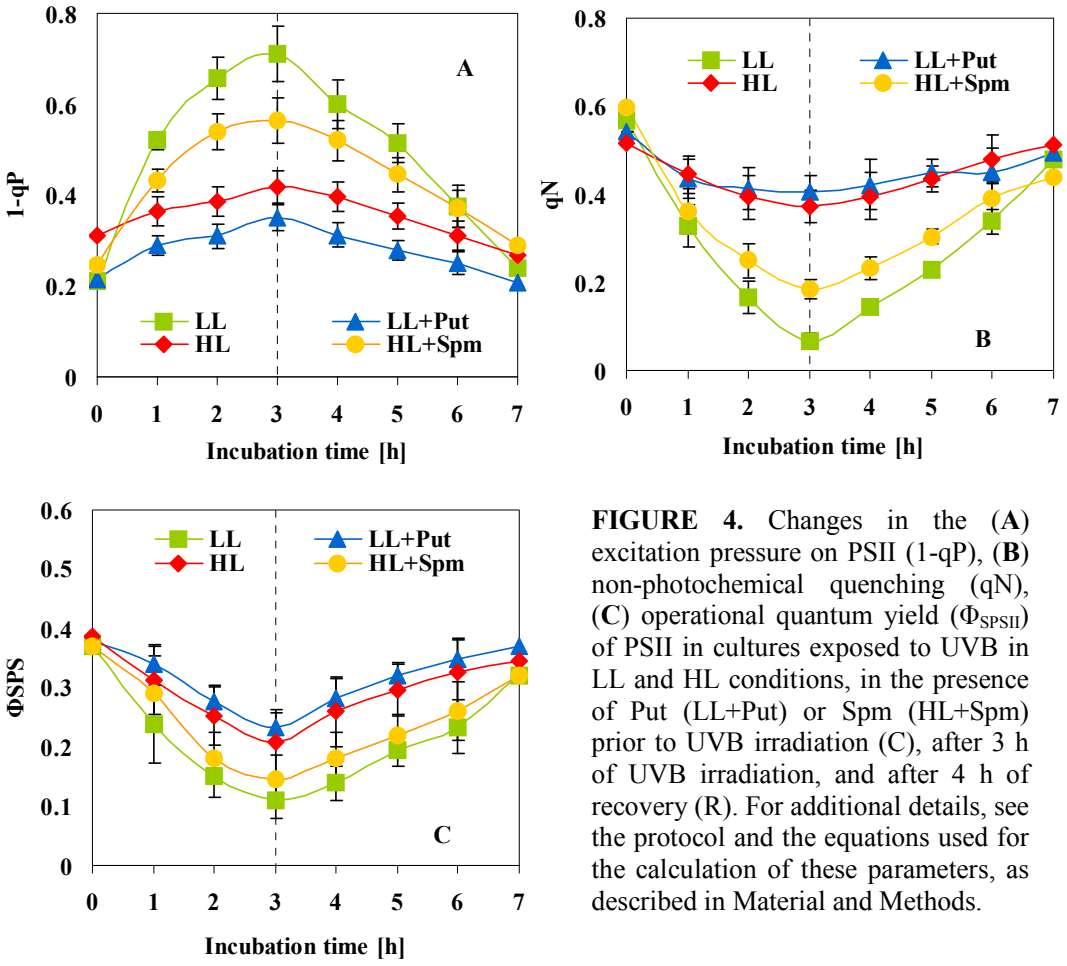


**FIGURE 4.** Changes in the (**A**) excitation pressure on PSII (1-qP), (**B**) non-photochemical quenching (qN), (**C**) operational quantum yield ($\Phi_{SPSII}$) of PSII in cultures exposed to UVB in LL and HL conditions, in the presence of Put (LL+Put) or Spm (HL+Spm) prior to UVB irradiation (C), after 3 h of UVB irradiation, and after 4 h of recovery (R). For additional details, see the protocol and the equations used for the calculation of these parameters, as described in Material and Methods.

The high excitation pressure induced by UVB in LL condition is largely diminished by Put (LL+Put+UVB). As the excitation pressure results from increases in the $Q_A^-/Q_A$ ratio, its reduction by Put confirms once more that Put by keeping the PQ pool oxidized antagonizes the increase induced by UVB in the excitation pressure reducing it by about 100% (Fig. 4A). On the contrary, the incubation of HL cultures with

Spm (HL+Spm+UVB) makes them more susceptible to the UVB action and due to the decreased capacity of photochemical quenching (also expressed by the over accumulation of $Q_A^-$), the excitation pressure exerted on PSII increases about 50% (Fig. 4A). The increase in the excitation pressure was accompanied by a decrease in the non-photochemical quenching capacity (qN), especially in LL+UVB conditions, but the addition of Put contributes to the reduction of this UVB effect by about 75%. Consequently, the cultures incubated in LL+Put+UVB and HL+UVB conditions exhibit an increased capacity for non-photochemical quenching as compared to the LL+UVB-culture. The addition of Spm increases the susceptibility to UVB radiation of the HL+UVB culture and qN decreases highly. The effect of Spm on the qN in HL+Spm+UVB culture is almost similar to that obtained for the LL+UVB culture (Fig. 4B). The investigation of qEmax showed that this is susceptible to the Spm treatment. This component of NPQ is more or less stable maintained during UVB irradiation treatment in LL-incubated cultures (treated or untreated with Put), whereas it increases slightly in HL +UVB conditions, an increase that is diminished by Spm (Fig. 5).

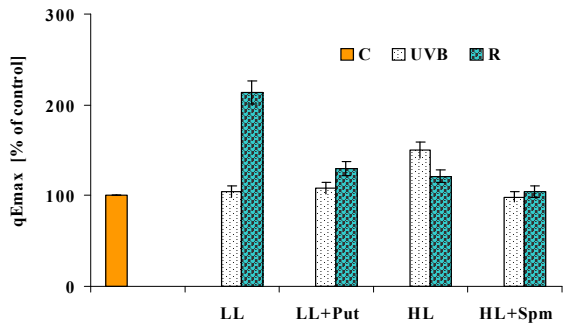


**FIGURE 5.** Changes in the energization quenching in culture supplemented or not with polyamines, after UVB irradiation and at recovery. For details, see text.

The final result of these changes on the quenching properties of the photosynthetic apparatus is the alteration in the operational quantum yield ($\Phi_{SPSII}$). This expression was found to exhibit a linear relationship with the quantum yield of carbon fixation (Genty et al., 1989) and, therefore, it gives an estimate of the fraction of PSII in the open state and the remaining fraction in the closed state. As it is depicted in Figure 4, the addition of exogenous polyamines influences the $\Phi_{SPSII}$ measured in LL+UVB and HL+UVB cultures. Under UVB irradiation, the reduction of $\Phi_{SPSII}$ in LL+UVB culture is reversed by Put (in LL+Put+UVB culture), whilst Spm acts synergistically with UVB and accentuates its inhibitory effect in the HL+Spm+UVB culture (Fig. 4C). Overall, Put

gives a response similar to that found in the HL+UVB-incubated culture, whereas Spm induces changes of the magnitude comparable to that found in LL+UVB-incubated culture. The ability to recover, as previously shown, is not affected by polyamines or PAR intensities and the cultures are able to restore the initial functional status (Fig. 4).

The changes described above indicate that Put and Spm can influence the sensitivity of the photosynthetic apparatus to UVB, which is in agreement with previously reported data that polyamines are involved in the photosynthetic apparatus response to abiotic stressors (Navakoudis et al., 2003; Sfichi et al., 2004; Sfakianakis et al., 2006). In cultures untreated with UVB, polyamines influence differently the photosynthetic behavior than in stress conditions. From the radar plots presented in Fig. 6, one can see that Put increases the density of active reaction centers of LL-incubated cultures, without affecting the photochemical and non-photochemical quenching, although it stimulates the energization quenching (qEmax). This last effect may be due to the intensification of the photosynthetic activity, as expressed by increased quantum efficiencies (Fv/Fm and $\Phi_{SPSII}$) and electron transport rate (PSIo). This photosynthetic burst induced by Put does not resulted from the increasing in the connectivity of the photosynthetic units (pG). In opposition, Spm induces increased photochemical and non-photochemical quenching capacity, probably due to the increase in the functional antenna size (ABS/RC).

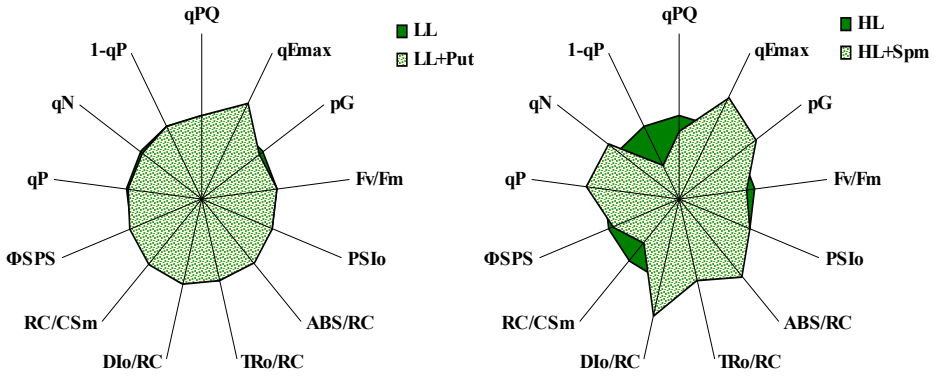


**FIGURE 6.** Spider plots with parameters calculated from chlorophyll fluorescence data. The original fluorescence curves have been normalized for minor chlorophyll concentration differences among the samples. All data are in arbitrary units. As reference values for the Put effect were used the values obtained for cultures incubated in LL conditions (*left)*. As reference values for the Spm effect were used the values obtained for cultures incubated in HL conditions (*right*).

Fewer centers are working when Spm is increased, but the high connectivity between them assures the efficiency of primary photochemistry rate (TRo/RC) and electron transport. The interesting fact is that Spm greatly increases the energization quenching (qEmax) and maybe this is the reason that the excitation pressure is kept low. Conclusively, Put enhances photosynthesis by activating the reaction centers, whilst Spm increasing the functional antenna size and contributes to the balance between energy captured and used in the photosynthesis. Neither Put, nor Spm show toxic effect on the photosynthetic apparatus functionality (Fig. 6).

### 1.1.4. Polyamine effect on the PSII heterogeneity in the photosynthetic apparatus exposed to UVB in LL and HL conditions

PSII heterogeneity in cultures treated or untreated with polyamines was investigated as previously described (*see* Material and Methods 8.3.).

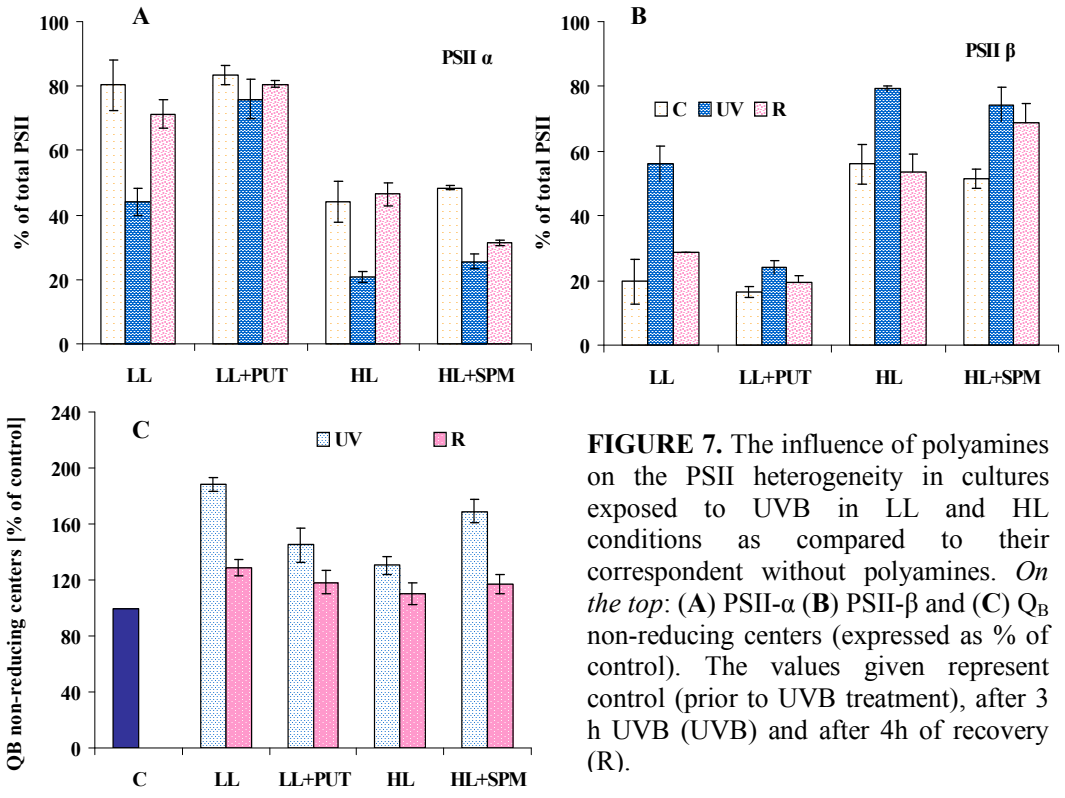


**FIGURE 7.** The influence of polyamines on the PSII heterogeneity in cultures exposed to UVB in LL and HL conditions as compared to their correspondent without polyamines. *On the top*: (**A**) PSII-α (**B**) PSII-β and (**C**) $Q_B$ non-reducing centers (expressed as % of control). The values given represent control (prior to UVB treatment), after 3 h UVB (UVB) and after 4h of recovery (R).

As one can see in Fig. 7A, cultures incubated in LL possess a higher level of functional PSII-α reaction centers than those maintained in HL conditions. It seems that HL increases the proportion of PSII-β reaction centers as compared to LL and this is not influenced by polyamine treatment (Fig. 7B). The treatment with UVB radiation induces an increase in PSII-β, parallel to a decrease in PSII-α and this effect is stronger in LL+UVB as compared to HL+UVB conditions. Put assures the protection of PSII-α in LL+Put+UVB culture, maintaining them in a functional state (Fig. 7A). Spm does not influence the UVB effect in HL+Spm+UVB culture and the functionality of PSII-α and PSII-β reaction centers is most similar with that found in HL+UVB culture (Fig. 7A-B).

Although the functional heterogeneity of PSII is influenced much more by Put than Spm, both polyamines exert a strong effect on the amount of $Q_B$ non-reducing centers. As compared to the LL+UVB culture, where about 80% of reaction centers are $Q_B$ non-reducing, the addition of Put (LL+Put+UVB culture) reduces the UVB effect on reaction centers by 30%. On the contrary, Spm amplifies the inactivating effect of UVB on the reaction centers in HL+Spm+UVB culture and there is an increase of 40% in the amount of $Q_B$ non-reducing centers as compared to the corresponding HL+UVB culture. Since Put preserves the functionality of PSII-α, it can be assumed that the lowering of the $Q_B$-non-reducing centers quantity, upon Put addition, is due to its effect on PSII-α, whilst Spm effect is related to the inactivation of both PSII-α and PSII-β reaction center functionality (Fig. 7C).

### 1.1.5. Polyamine impact on the maximal net photosynthetic rate in the LL- and HL-adapted cultures under UVB irradiation

In parallel to the above mentioned effects, the exposure to UVB radiation led to a decrease in the maximal net photosynthetic rate (Fig. 8). This response was strongly influenced by exogenous polyamines. Compared to the corresponding control cultures, the maximal net photosynthesis values obtained for the cultures treated with UVB in LL and HL conditions were reduced about 54% and 27%, respectively. In opposition, Put-treated culture (LL+Put+UVB) behave with less UVB sensitivity showing 76% better photosynthetic rate than the LL+UVB culture, while the addition of Spm in HL+UVB culture (HL+UVB+Spm) enhances the decrease of the photosynthetic rate by about 43%. The decline induced by UVB in the maximal net photosynthetic rate in LL- and HL-

conditions rapidly recovers to a significant level (between 80% and 95% for all treatments) after the cessation of UVB treatment (Fig. 8).

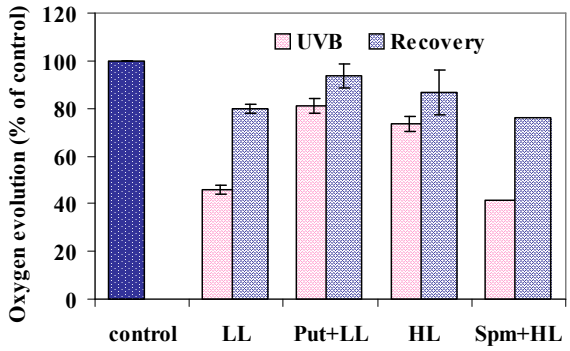


**FIGURE 8.** Oxygen evolution in cultures exposed to UVB under LL and HL conditions, with and without exogenous polyamines. The values represent the means ± SD of three to five samples and are expressed in % of the corresponding control values.

## 1.2. POLYAMINE PATTERN REGULATES THE LHCII STATUS AND THROUGH THIS THE PHOTOSYNTHETIC APPARATUS RESPONSE TO UVB

Previously, it was shown that UVB induced alterations in the pattern of pigments and polyamines, as well as, in the structural organization of LHCII. The most important finding concerning the UVB effect on the molecular structure of the photosynthetic apparatus was the fact that an increase in the oligomeric form of LHCII is accompanied by a decrease in the thylakoidal Put/Spm ratio. Therein, it was suggested that changes in the pattern of thylakoid associated polyamines induce changes in the LHCII oligomerization status that triggers the entire reaction cascade constituting the response of the photosynthetic apparatus to UVB (*see* Results and Discussion-*Chapter I*). In this context, it is important to see how the manipulation of polyamine pattern affects the LHCII status. The data obtained are described below.

### 1.2.1. UVB-induced alterations in the Put/Spm ratio in isolated thylakoids and LHCII sub-complexes

Polyamines were qualitatively and quantitatively determined by HPLC in isolated thylakoids membranes, as well as in LHCII forms (*see* Material and Methods). The thylakoid membranes and subsequently the LHCII oligomeric and monomeric forms were isolated from cultures prior to UVB irradiation, after 3 h of UVB irradiation and after an additional 4 h period without UVB treatment.

As it is shown in Fig. 9, the Put content in thylakoids decreases during UVB irradiation reaching values significantly lower than those of the corresponding control samples. The intensity of this UVB-induced effect is highest in LL+UVB culture (46% below the control) and lowest in LL+Put+UVB culture (12% below the control level). On the contrary, Spm amount increases after UVB treatment. Spm reaches the highest values in LL+UVB (73% over the control value) and HL+Spm+UVB (60% over the control) conditions. Although the total polyamine content is not significantly changed by UVB exposure (Fig. 10A), the alterations induced by UVB in the pattern of Put and Spm associated to the thylakoid membranes lead to the decrease of Put/Spm ratio during the UVB treatment. Compared to the corresponding control values, the lowest Put/Spm ratios are in LL+UVB and HL+Spm+UVB cultures, while the highest value is found in LL+UVB+Put-culture (Fig. 10B). After the UVB irradiation, the Put/Spm ratios partially recover in all treatments.

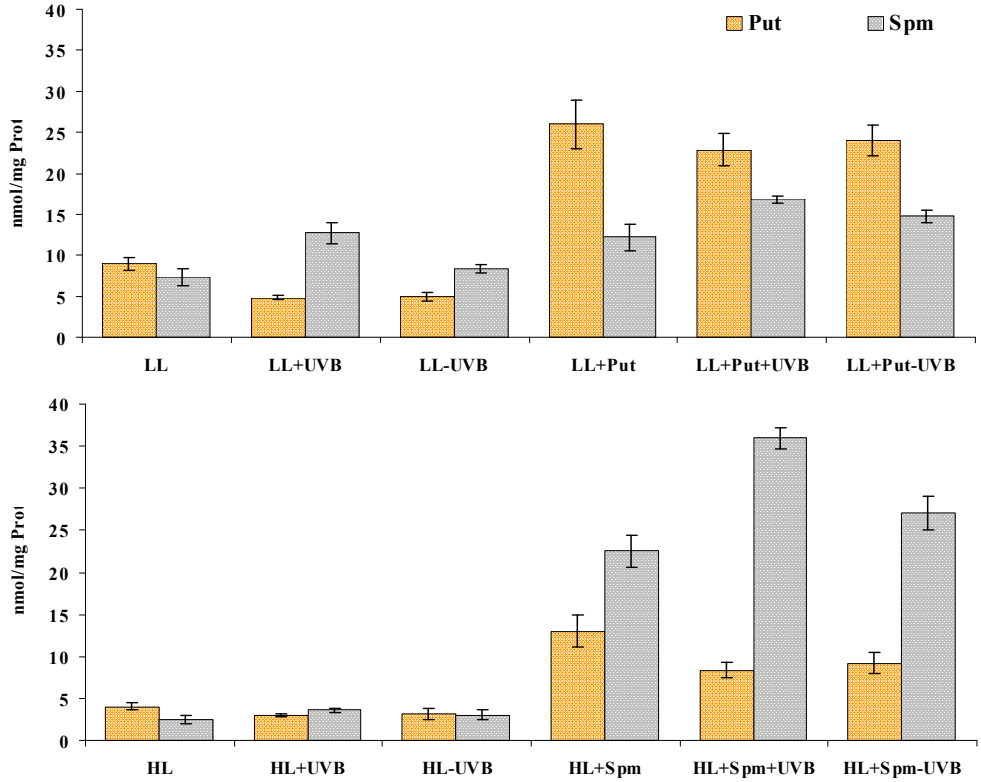


**FIGURE 9.** Polyamine content in thylakoids isolated from LL- and HL-cultures treated or not with exogenous polyamines, prior to UVB irradiation, after 3h of UVB irradiation (+UVB) and after 4 h of recovery (-UVB).

Oscillations in the pattern of thylakoid-associated polyamines are followed by similar alterations in the Put/Spm ratio in the oligomeric/monomeric forms of LHCII (Fig. 11). Distinctly lower Put/Spm ratios are found in LL+UVB and HL+Spm+UVB conditions. Here, the Put/Spm ratio in LHCII oligomers decreases about 70% and 60%, respectively. Put inhibits the UVB effect in LL conditions and determines an increase in the Put/Spm of 55% in the LL+Put+UVB culture, as compared to the value obtained for LL+UVB culture. The Put/Spm values also decrease by 20% in HL+UVB culture. This effect is amplified by Spm about 40% (HL+Spm+UVB culture). Decreasing the Put/Spm ratio in LHCII oligomers, it increases in LHCII monomers. Put/Spm ratio exhibits high values in the monomeric fraction of LHCII isolated from LL+UVB-culture (about 160% of control). Put attenuates this increase in the LL+Put+UVB culture, giving an effect similar to that found in HL+UVB culture. Upon Spm addition, the Put/Spm ratio value in the monomeric fraction of LHCII increases with 50% in the HL+Spm+UVB culture (Fig. 11).

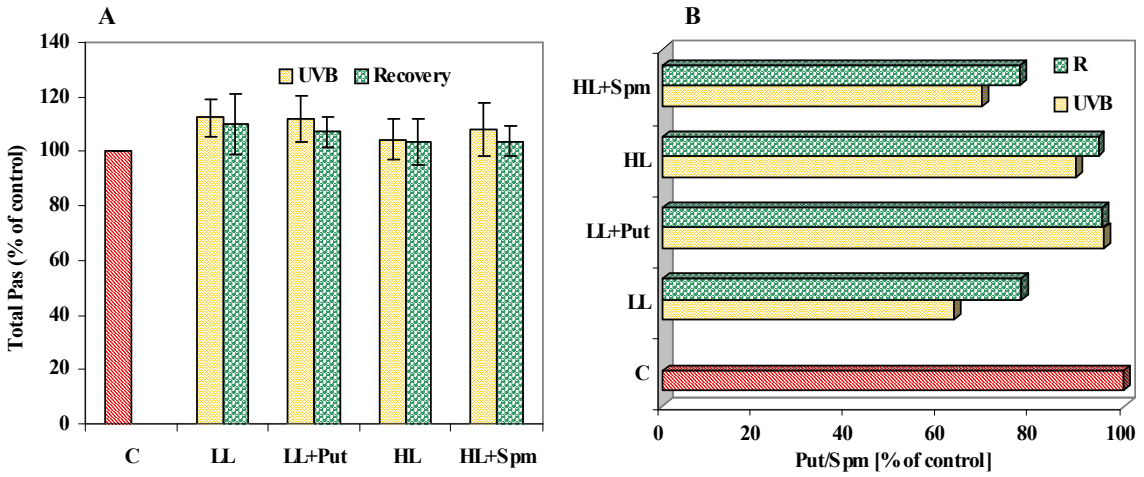


**FIGURE 10.** Changes in the (**A**) total polyamine content and Put/Spm ratio in thylakoid membranes under UVB irradiation and recovery, in culture incubated in LL or HL in the presence or not of exogenous polyamines (LL+Put; HL+Spm) The values are expressed in % of the values obtained for corresponding control cultures.

A different pattern of changes was obtained for the cellular polyamines. The Put/Spm ratio is not significantly changed upon UVB treatment and this suggests that the effect of UVB on the antenna size is not mediated through changes in the cellular

polyamines, since they exhibited a different response to UVB as compared to antenna (data not shown).

Overall, the pattern of changes in the Put/Spm ratio in thylakoids is similar to that found in the oligomeric LHCII fraction and this indicates that the exogenous polyamines are bound to thylakoids, in general, and LHCII fractions, in particular. Therefore, one can assume that the reduction or the enhancing of UVB effect in specific conditions of illumination is the direct consequence of the polyamine pattern alteration. The most striking evidence for the above affirmation was provided by the results obtained from the investigation of changes occurring in the LHCII structure.

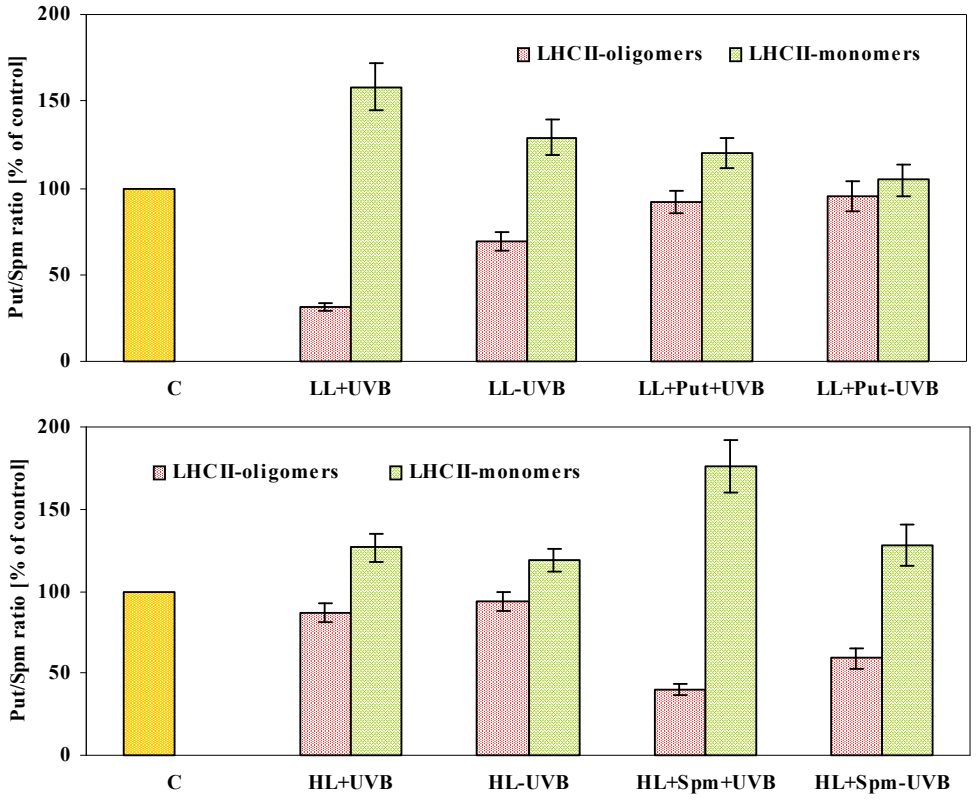


**FIGURE 11.** Alterations in the Put/Spm ratio in the oligomeric and monomeric fractions of LHCII, under UVB irradiation and recovery, in culture incubated in LL or HL in the presence or not of exogenous polyamines (LL+Put; HL+Spm). The values are expressed in % from the values obtained for corresponding control cultures.

### 1.2.2. Regulation of LHCII oligomerization by polyamines under UVB exposure and recovery

The photosynthetic sub-complexes and, mainly, the monomeric and oligomeric forms of LHCII were separated from the isolated thylakoid membranes prior to UVB irradiation, after 3 h of UVB irradiation and after an additional 4 h period without UVB. Quantitative analysis of the isolated LHCII sub-complexes revealed that the UVB treatment caused an increase in the oligomeric fraction simulatneously to the decrease in the monomeric fraction (Fig. 12A-B).

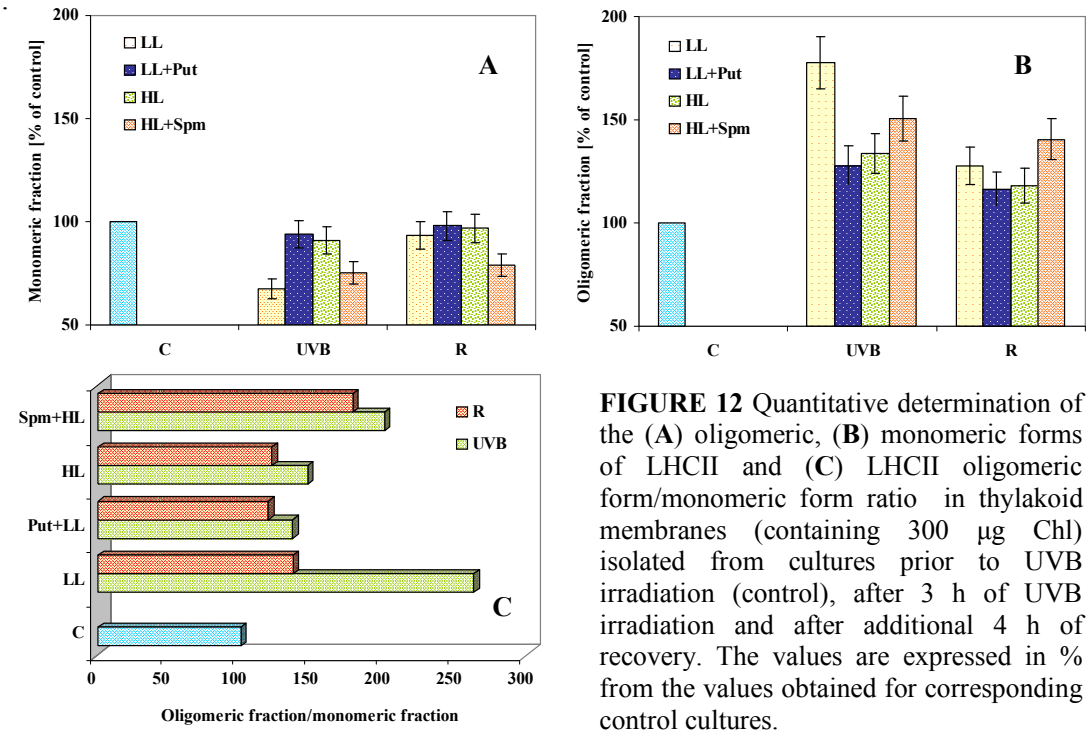


**FIGURE 12** Quantitative determination of the (**A**) oligomeric, (**B**) monomeric forms of LHCII and (**C**) LHCII oligomeric form/monomeric form ratio in thylakoid membranes (containing 300 μg Chl) isolated from cultures prior to UVB irradiation (control), after 3 h of UVB irradiation and after additional 4 h of recovery. The values are expressed in % from the values obtained for corresponding control cultures.

Compared to the corresponding control values, the UVB-induced increase in the oligomeric fraction is higher in LL (77%) than in HL culture (33%). Exogenously supplied Put to LL culture (LL+Put+UVB) leads to a decrease of about 50% in the oligomeric fraction of LHCII as compared to the values obtained for the LL+UVB treatment. In opposition, exogenously supplied Spm to the HL culture (HL+Spm+UVB) causes an increase of about 80% in the oligomeric fraction, as compared to the data obtained for the HL+UVB culture. These changes are rapidly recover to a great extent within 4 h after the end of UVB irradiation (Fig. 12A-B). The increase of the LHCII

oligomeric fraction combined with the decrease in the monomeric fraction have as result an increase in the oligomeric/monomeric fraction ratio during UVB exposure (Fig. 12C). In LL conditions, this ratio increases about 163% under UVB radiation, but exogenously supplied Put reduces this increase to 35%. In HL conditions, the UVB treatment increases this ratio to about 46%, but an elevated content of Spm (obtained after Spm addition) enhances this increase to 100%. Within 4 h from the cessation of UVB treatment, the oligomeric forms of LHCII decreases (Fig. 12A) simultaneously to the increase in the corresponding monomeric ones (Fig. 12B) and the oligomeric/monomeric fraction ratio partially recovers (Fig. 12C). The above data shows clearly that the composition of the LHCII (given by the oligomerization status of LHCII) can be changed by the manipulation of polyamine pattern with exogenous Put or Spm. This is the most important evidence in support of the hypothesis that Put/Spm ratio in thylakoids regulates the entire photosynthetic behavior to UVB radiation through changes in the LHCII size.

### 1.3. POLYAMINE INFLUENCE ON THE PIGMENT POOL UPON UVB IRRADIATION

The pattern of Chl and Car was investigated in the cultures treated with polyamines. The determination of Chl a/b shows that there is a strict correlation between the changes observed in LHCII size and the Chl a/b values (Fig. 13A). The values calculated for Chl a/b ratios are lowered by Spm (in HL+Spm+UVB culture) as compared to HL+UVB culture. This demonstrates again that Spm induces the increase in the LHCII size, since Chl a/b ratio is a factor indicating the LHC II antenna size (Anderson et al., 1988). In contrast, Put increases the Chl a/b in the LL+Put+UVB culture, as compared to LL+UVB culture, due to its inhibitory effect on LHCII size increasing (Fig. 13A). At recovery, Chl a/b ratio increases to control level in all cultures (Fig. 13A). The fluctuations of Chl a/b ratios in UVB-irradiated *wt* cultures, although not very significant quantitatively, are important because they are related to the oscillations in antenna size. The changes that occurred in Chl content (data not shown), as well as in Chl a/b ratio indicate that cultures show a highly adaptive behavior to the conditions applied during experiments (LL and HL conditions).

Examining the pattern of Car, it is found that Put addition induces an increase in loroxanthin (Lx) accompanied by a decrease in lutein (L), which results in an increased

Lx/L ratio values. This means that Put stimulates the conversion of lutein (L) in loroxanthin (Lx) in LL+Put+UVB culture (Fig. 13B). On the contrary, the conversion of violaxanthin (Vx) to zeaxanthin (Zx) is highly inhibited by Put, although there is a stimulation of antheraxanthin (Ax) synthesis, which leads to a small increase in the β-xanthophyll pool (Fig. 13B), as compared to LL+UVB-culture. In contrast to Put, Spm stimulates the production of L, maybe due to its effect on the stabilization of LHCII complexes but decreases the β-xanthophyll pool, as well as, the Vx conversion but in a smaller extent as Put (Fig. 13 B-C).

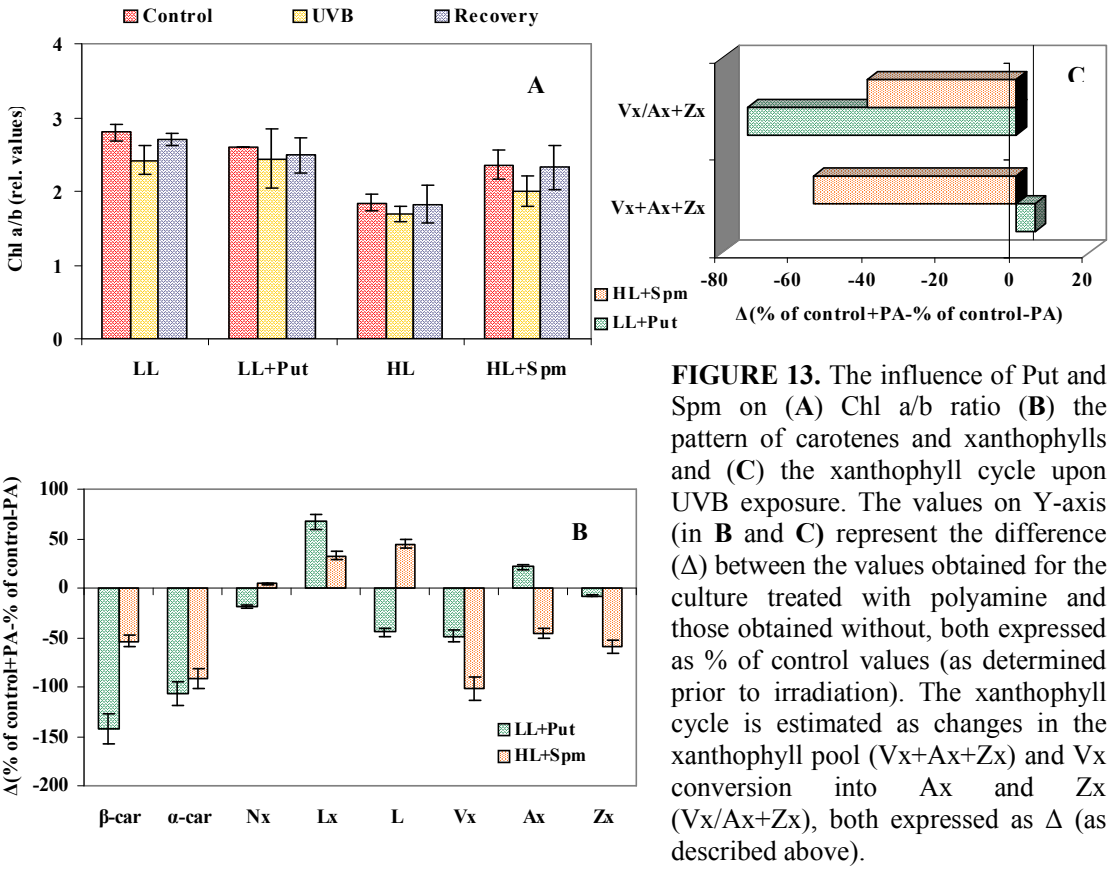


**FIGURE 13.** The influence of Put and Spm on (**A**) Chl a/b ratio (**B**) the pattern of carotenes and xanthophylls and (**C**) the xanthophyll cycle upon UVB exposure. The values on Y-axis (in **B** and **C)** represent the difference (Δ) between the values obtained for the culture treated with polyamine and those obtained without, both expressed as % of control values (as determined prior to irradiation). The xanthophyll cycle is estimated as changes in the xanthophyll pool (Vx+Ax+Zx) and Vx conversion into Ax and Zx (Vx/Ax+Zx), both expressed as Δ (as described above).

## 1.4. ROS GENERATION IN THE CULTURES EXPOSED TO UVB IN DIFFERENT EXPERIMENTAL CONDITIONS

It is possible that the inactivation of reaction centers, like occurred in the above-described situations, resulted from ROS accumulation. Previous experimental data suggest that the oxidative damage may induce conformational changes in the reaction

centers, which could serve as a triggering or sensing signal for the primary proteolytic cleavage (Ohad et al., 1985). UVB radiation has been shown to stimulate the generation of ROS in different conditions (Rao et al., 1996; He and Häder, 2002). The origin of these ROS is unclear but it has been proposed that UVB exposure may lead to ROS generation, by increasing NADPH oxidase activity (Rao et al, 1996). It is proposed that ROS mediate a series of signal transduction pathways each controlling the expression of different specific genes, i.e. up-regulation of pathogenesis-related genes and down-regulation of photosynthetic genes. Consequently, it has been concluded that the antioxidant capacity of a plant tissue dictates the relative sensitivity of photosynthetic genes to UVB induced down-regulation (Surplus et al., 1998).

To check if ROS can represent the signal that induce changes in the Put/Spm ratio in thylakoids (and LHCII) which in turn induce the increase of LHCII size and all the cascade of reactions exhibited to UVB, ROS were measured in the cultures treated with and without polyamines, prior and after 3h of UVB treatment.

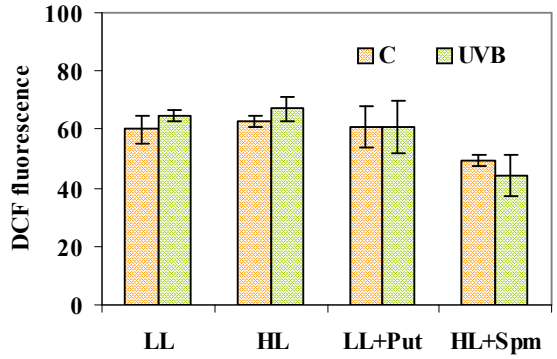


**FIGURE 14.** ROS generation in cultures incubated in LL or HL conditions, supplemented or not with exogenous polyamines, as determined using a method based on the determination of DCF fluorescence, prior (C) and after 3h UVB irradiation (UVB)

ROS was first detected by using dichlorofluorescin diacetate (DCFH-DA) (*see* Material and Methods 10.1.). This non-polar compound is converted to the polar derivative DCFH by cellular esterases when it is taken up. DCFH is nonfluorescent but highly fluorescent when oxidized to dichlorofluorescein (DCF) by intracellular ROS and other peroxides (He and Häder, 2002). This method permit *in vivo* assessment of ROS and it is widely used as a non-invasive and sensitive method for the determination of ROS. As depicted in Figure 14, UVB radiation did not have a significant effect on ROS generation. In order to check the above results, ROS was quantified using the

chemiluminescence assays for the detection of $H_2O_2$ and $O_2^{\cdot-}$ (*see* Material and Methods 10.2.) in thylakoid membranes isolated from LL incubated cultures, which exhibited the strongest effect to UVB radiation. The slight increase in the production of $H_2O_2$ found after 3h of UVB irradiation cannot account for the strongest effect of UVB on the structure and function of the photosynthetic apparatus found in these illumination conditions. This means that ROS is not a signal for the chain of reactions triggered by UVB (Fig. 15).

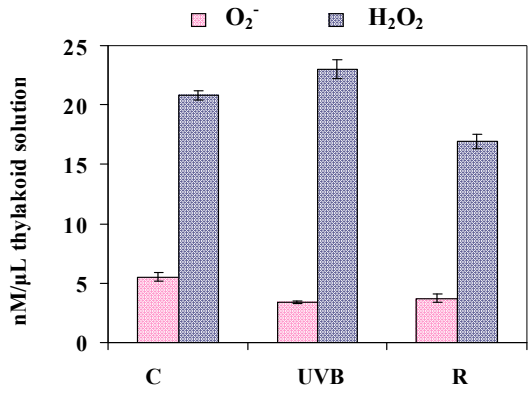


**FIGURE 15.** ROS (anion superoxide, $O_2^{\cdot-}$ and hydrogen peroxide, $H_2O_2$ generation in thylakoid membranes isolated from LL-incubated culture at three experimental steps: prior to UVB irradiation (control), after 3h of UVB irradiation (UV) and after recovery.

## 2. THE INFLUENCE OF POLYAMINES ON THE *wt-lhc*- PHOTOSYNTHETIC APPARATUS SENSITIVITY TO UVB

The data presented above demonstrates that two factors are important for the determination of photosynthetic apparatus sensitivity to UVB radiation: LHCII/thylakoid-associated polyamines and LHCII size. The latter also determines the recovery capacity (*see* Results and Discussion-*Chapter II*). The former can regulate the latter, which demonstrates that polyamines regulate the entire reaction cascade that constitutes the response of the photosynthetic apparatus to UVB. Consequently, polyamines may represent the primary target of the UVB radiation in the photosynthetic apparatus.

To verify this hypothesis, the involvement of polyamines in the determination of the degree of sensitivity to UVB of a mutant photosynthetic apparatus without LHCII was investigated. This was made by comparing the response of the *wt-lhc* photosynthetic apparatus to UVB in function of the presence or absence of exogenously supplied Put and Spm.

## 2.1. POLYAMINE INFLUENCE ON THE BIOENERGETICS OF *wt-lhc* PHOTOSYNTHETIC APPARATUS EXPOSED TO UVB

### 2.1.1. Changes in the Chl fluorescence quenching determined by addition of exogenous polyamines in *wt-lhc* cultures

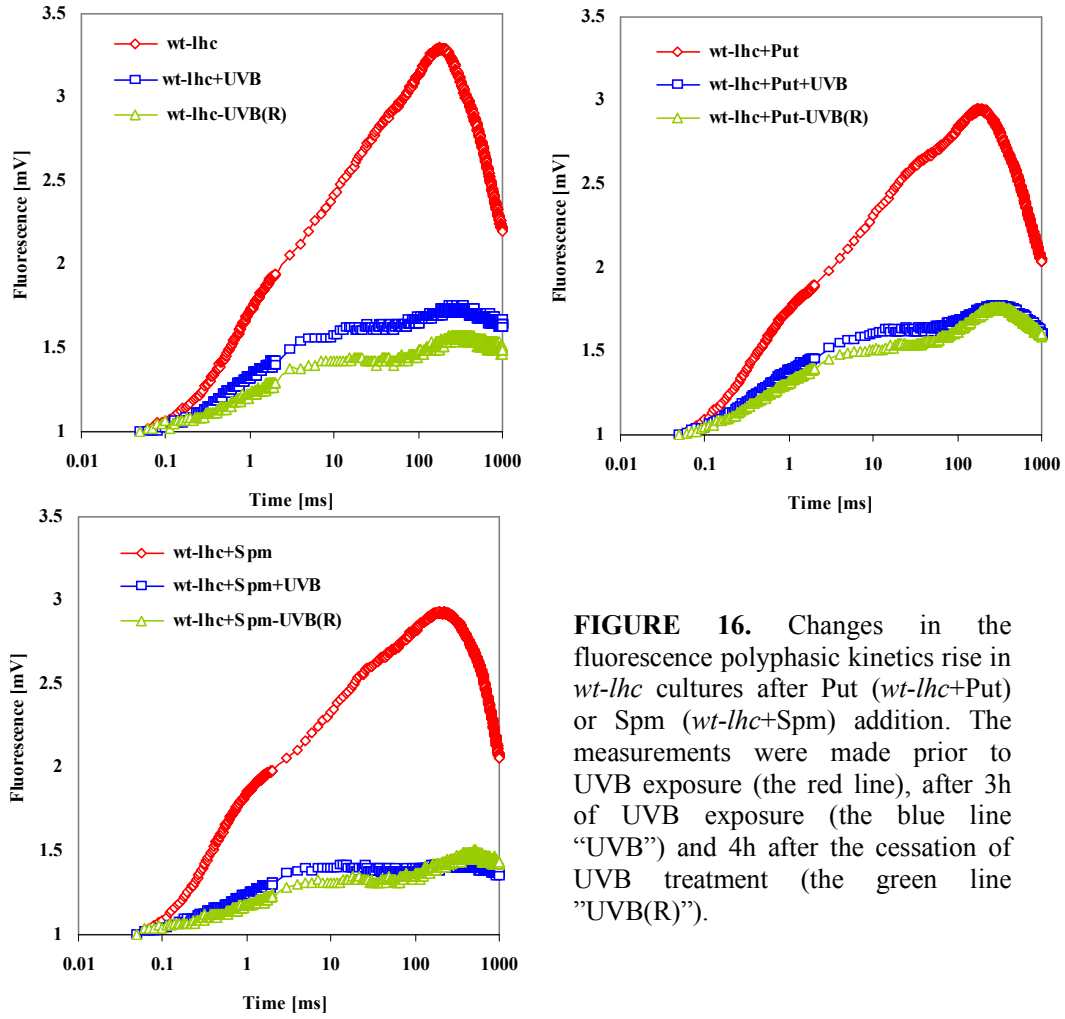


**FIGURE 16.** Changes in the fluorescence polyphasic kinetics rise in *wt-lhc* cultures after Put (*wt-lhc*+Put) or Spm (*wt-lhc*+Spm) addition. The measurements were made prior to UVB exposure (the red line), after 3h of UVB exposure (the blue line "UVB") and 4h after the cessation of UVB treatment (the green line "UVB(R)").

The shape of O-J-I-P transients showed changes upon UVB irradiation, which are intensified or diminished in relation to the polyamine treatment. In Figure 16 it is shown that the addition of Put and Spm in *wt-lhc* cultures results in the decrease of fluorescence yield, even at the control level. Upon UVB exposure, the shape of transients is highly affected, especially in the culture treated with Spm, as well as, in the culture untreated with polyamines. This loss in Chl fluorescence denotes that PSII functionality is strongly impaired by UVB. Between the polyamines used, Put seems to confer some degree of

protection against UVB damage (Fig. 16), because the decline of Fm is weaker in *wt-lhc*+Put+UVB than in Spm-treated culture (*wt-lhc*+Spm+UVB) or in the culture untreated with polyamines (*wt-lhc*+UVB). Recovery did not occur in any of the investigated cultures, where the functionality of PSII is irreversible damaged by UVB (Fig. 16). As compared to the results obtained for the *wt*-photosynthetic apparatus, the data showed here highlights the importance of LHCII in the sensitivity to UVB and, mainly, in the promotion of the recovery mechanisms.

From the Chl fluorescence data, several parameters proved to be important for the estimation of polyamine regulatory role in the *wt*-photosynthetic apparatus sensitivity to UVB were also calculated for the *wt-lhc* cultures, supplied or unsupplied with exogenous polyamines. The quenching of Fm due to the harmful effect of UVB radiation, as well as, the absence of recovery demonstrates that the in the mutant photosynthetic apparatus PSII is irreversibly damaged by UVB (Fig. 16). The values obtained for Fv/Fm, as an indicator of the PSII functionality, show that Spm amplifies the UVB effect giving an additional reduction of 40%, as compared to Fv/Fm values obtained for *wt-lhc*+UVB or *wt-lhc*+Put+UVB where Fv/Fm decreased to approximately 60% of the control (Fig. 17A). This effect seems to result from the inactivation of active reaction centers upon UVB. The inactivated reaction centers (RC/CS) are increased by 75% in *wt-lhc*+Put+UVB and 90% in *wt-lhc*+Spm+UVB (Fig. 17B). This reduction in density of active reaction centers leads to an increase in the functional antenna size (ABS/RC) from 175% in the *wt-lhc*+UVB culture to 225% in the *wt-lhc*+Spm+UVB culture. Put reduces this effect to 150% (*wt-lhc*+Put+UVB) (Fig. 17C). Similarly, the dissipation of excess energy (DIo/RC) increases proportionaly to the functional antenna size (Fig. 17D). After the cessation of UVB treatment, none of the above parameters recovered from the UVB effect, demonstrating once more that the restoration of the photosynthetic apparatus to the initial status after UVB is strictly dependent on the LHCII.

Overall, polyamine addition exerts a slight influence on the magnitude of response induced in the *wt-lhc*+UVB culture. There is a slight protective effect of Put, whilst Spm accentuates the UVB effect, but the differences registered between the amplitude of responses exhibited to UVB in the experimental variants used are quite small as compared to those found in *wt* between the polyamine treated and untreated

cultures. Thus, these results demonstrate that only in the presence of LHCII can polyamines closely regulate the photosynthetic apparatus sensitivity to UVB.

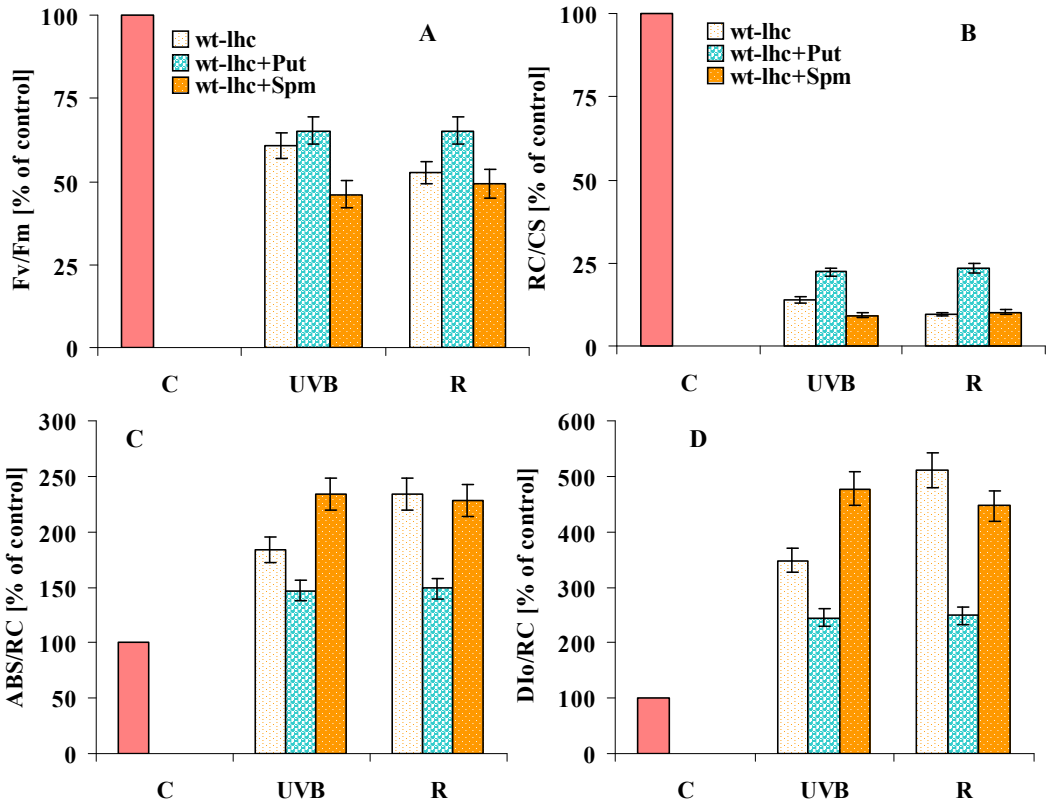


**FIGURE 17.** Changes in the (**A**) photosynthetic efficiency ($F_v/F_m$), (**B**) active reaction center density (RC/CS), (**C**) functional antenna size (ABS/RC) and (**D**) rate of dissipation energy per reaction center ($DI_0$/RC), in *wt-lhc* cultures incubated or not with Put (*wt-lhc*+Put) or Spm (*wt-lhc*+Spm). The values are expressed in percentage from the corresponding control (considered 100%).

The inactivated reaction centers were quantified with DCMU, as previously described (*see* Material and Methods 8.3.). UVB exposure induces 50% increases in the amount of $Q_B$ non-reducing centers. Put reduces this effect and the reaction centers unable to reduce $Q_B$ decreases to 27%. In contrast, Spm amplifies the UVB effect and the $Q_B$ non-reducing centers increases to 60% in the *wt-lhc*+Spm culture (Fig. 18). By comparing the percentage obtained for the inactivation of reaction centers (RC/CS) to that obtained for $Q_B$ non-reducing centers, the resulted difference can be attributed to the fact that a part of reaction centers proved to be also unable to reduce $Q_A$. Thus, in *wt-lhc*

cultures, independently of the polyamine treatment, UVB induces a blockage in the electron transfer beyond $Q_A$.

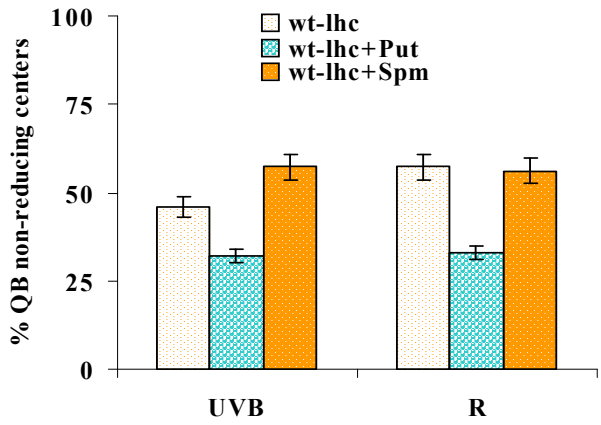


**FIGURE 18.** Polyamine influence on the UVB-induced increase in the amount of $Q_B$ non-reducing centers in the *wt-lhc* culture and the potential of cultures treated with Put (*wt-lhc*+Put) or Spm (*wt-lhc*+Spm) to recover from the UVB effect, as compared to the culture not treated with polyamines (*wt-lhc*).

The investigation of electron transport kinetics using the parameter PSIo, which describes the efficiency of the movement of an electron further than $Q_A^-$ in the electron transport chain, showed that the inhibition of electron transport by UVB is slightly affected by polyamines. The rate of electron transport inhibition ranges from 70% in the *wt-lhc*+UVB and *wt-lhc*+Put+UVB cultures to 60% in wt-lhc+Spm+UVB. As previously described for the other parameters, recovery does not occur in any of the investigated cultures demonstrating that the PSII functionality is irreversibly affected (Fig. 19).

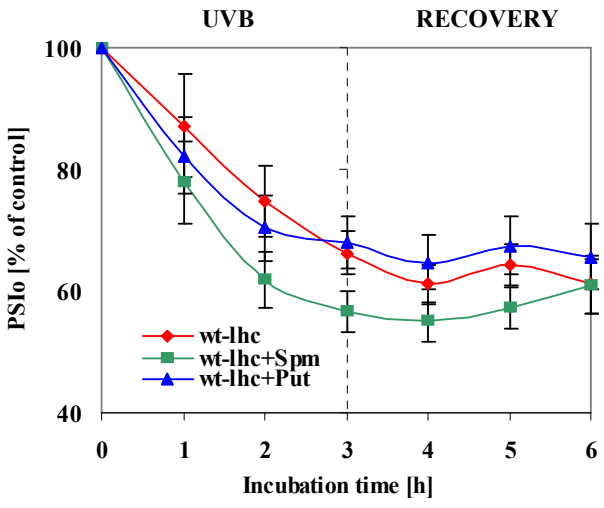


**FIGURE 19.** Kinetics of electron transport efficiency after $Q_A^-$ (PSIo) in the culture treated with Put (*wt-lhc*+Put) and Spm (*wt-lhc*+Spm) as compared to the not treated one (*wt-lhc*). The values are expressed as percentage from the initial values (measured at the beginning of UVB treatment).

The perturbations of electron transport induce a decrease in the photochemical quenching capacity (Fig. 20A). As result, there is an over accumulation of reduced $Q_A$, due to the imbalance between the excitons trapped in the reaction centers (TRo/RC) and those used to drive the electron transport (data not shown). Consequently, the excitation pressure exerted on PSII increased (Fig. 20B).

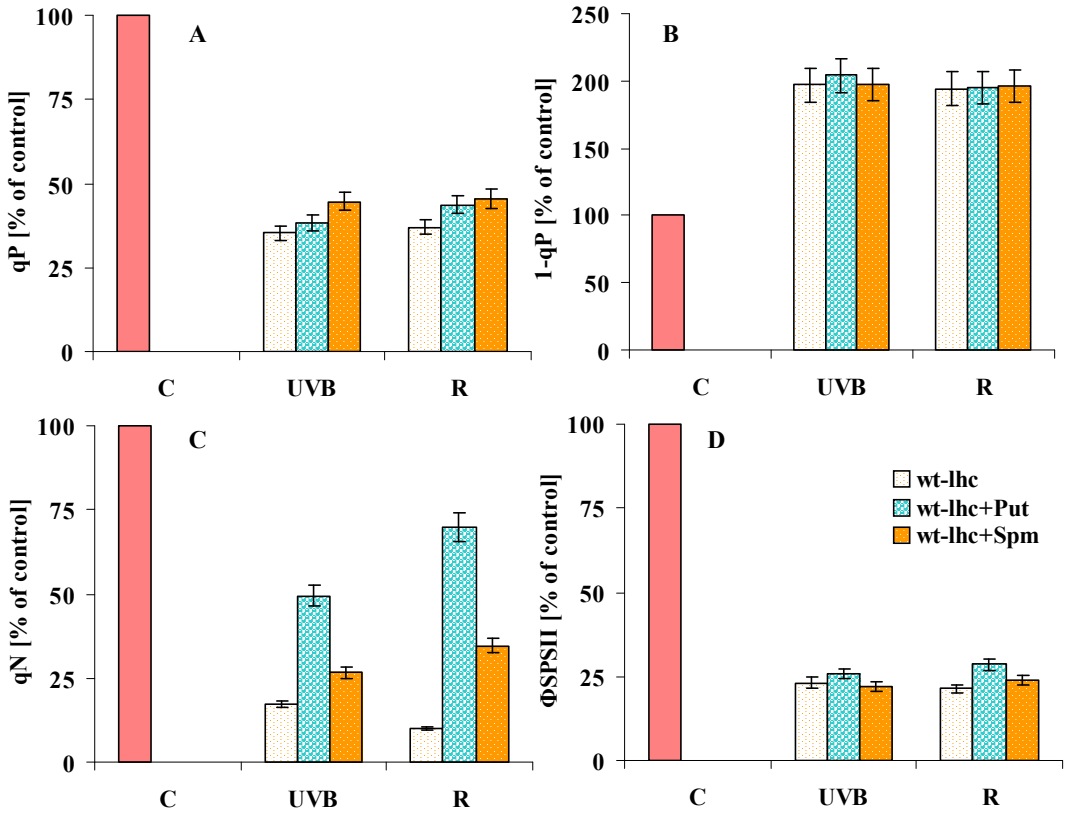


**FIGURE 20.** Alterations in the (**A**) photochemical quenching (qP), (**B**) excitation pressure (1-qP), (**C**) non-photochemical quenching (qN) and (**D**) operational quantum yield of PSII ($\Phi_{SPSII}$) in *wt-lhc* cultures and those treated with Put (*wt-lhc*+Put) and Spm (*wt-lhc*+Spm), after 3h of UVB irradiation and an additional 4h incubation in conditions without UVB. The values are given in % of the correspondent control (100%).

There are no significant differences between the changes induced by UVB in the *wt-lhc* culture and those observed in the cultures supplemented with polyamines. The excitation pressure is similarly increased by 200% over the control values in all cultures. As a consequence, the operational quantum yield of PSII ($\Phi_{SPSII}$), which gives a measure of PSII functionality, is decreased to 25% of control in all cultures (Fig. 20D). In contrast, the values obtained for qN were higher in the cultures treated with polyamines than in *wt-lhc*+UVB culture (Fig. 20C). Independent of the experimental conditions

applied, none of the cultures recovered from the UVB effects. This demonstrates once more that a photosynthetic apparatus without LHCII has no potential to restore its functionality (Fig. 20).

The above data demonstrated that UVB radiation induced similar modifications in the *wt-lhc* photosynthetic apparatus as in the *wt* one, but polyamines do not exert a strong influence on the degree of sensitivity to UVB as they do in *wt*+UVB-cultures, nor contribute to the recovery of the photosynthetic apparatus to the initial status. In a *wt*-photosynthetic apparatus, Put acts differently of Spm (Fig. 6), as compared to a *wt-lhc* photosynthetic apparatus where both polyamines show similar responses (Fig. 21). This demonstrates that one of the roles played by polyamines in cells is to regulate the response to environmental factors by adjusting the size of LHCII. Ultimately, this means that polyamines are regulators of the adaptation and acclimation of the photosynthetic apparatus to environment.

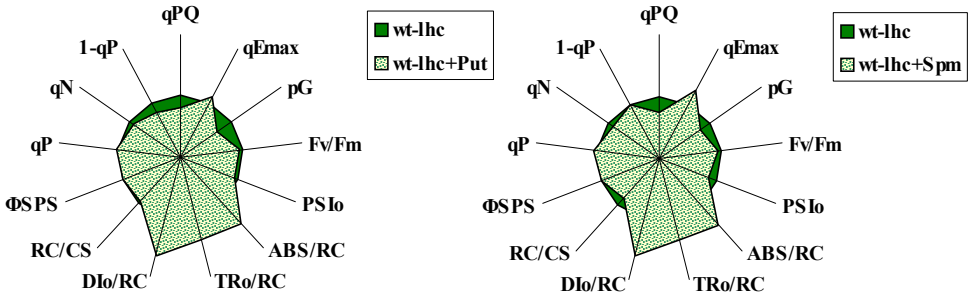


**FIGURE 21.** Spider plots of parameters calculated from chlorophyll fluorescence data. The original fluorescence curves have been normalized for minor chlorophyll concentration differences among the samples. All data are in arbitrary units. The data for control treatment (*wt-lhc*) has been used as a reference for Put (*left: wt-lhc*+Put) and Spm effect (*right: wt-lhc*+Spm).

### 2.1.2. Changes in the maximal net photosynthetic rate of *wt-lhc* cultures upon polyamine addition

The changes occurring in photosynthetic apparatus bioenergetics have as overall result a decrease in the maximal photosynthetic rate (oxygen evolution) of the cultures exposed to UVB treatment (Fig. 22). In *wt-lhc*+UVB cultures, the oxygen evolution rate decreased to 40% of the correspondent control, the addition of Put increased the oxygen evolution by 20%. None of the investigated culture restored its ability for oxygen evolution after the cessation of UVB treatment, fact demonstrating the irreversibility of changes induced by UVB in the photosynthetic apparatus in the absence of LHCII.

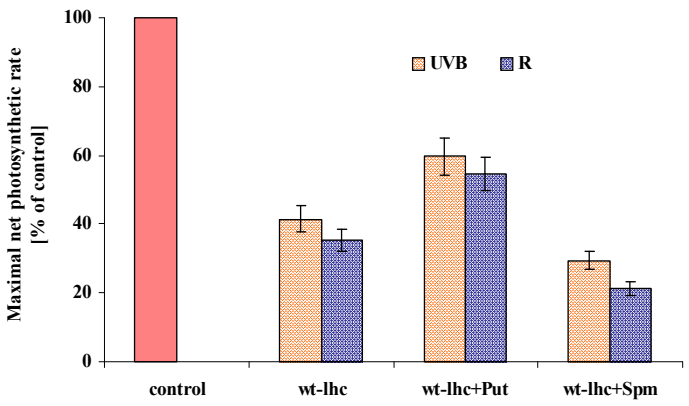


**FIGURE 22.** Maximal net photosynthetic rate in *wt-lhc* cultures treated or not with exogenous Put (*wt-lhc*+Put) or Spm (*wt-lhc*+Spm) upon UVB irradiation and recovery (expressed as % of control).

## 2.2. BIOCHEMICAL ALTERATIONS IN THE PHOTOSYNTHETIC APPARATUS UPON UVB TREATMENT AND THEIR INFLUENCE BY POLYAMINES

For the *wt* cultures, it was shown that alterations in the pattern of LHCII/thylakoid associated Put and Spm induced inverse related changes in the LHCII antenna size. Thus, it is interesting to see what modifications occur in the pattern of thylakoid-associated polyamine when the LHCII is absent.

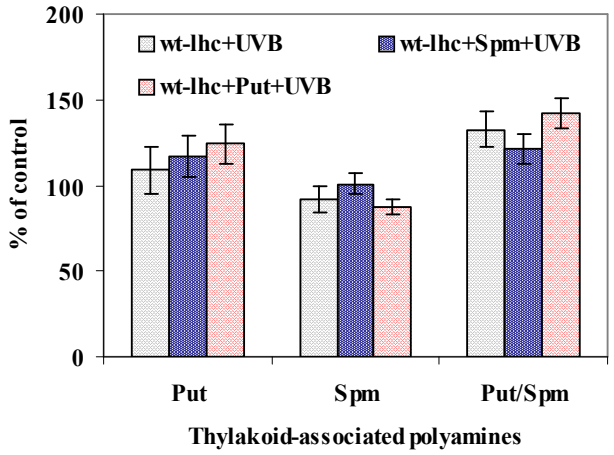


**FIGURE 23.** Polyamine (Put and Spm) content and the Put/Spm ratio in thylakoids isolated from *wt-lhc*, *wt-lhc*+Put and *wt-lhc*+Spm cultures after 3h of UVB irradiation. The values are given as percentage from the corresponding control (100%).

As it is shown in Fig. 23, there is no significant difference between the pattern of thylakoid-associated polyamines in the *wt-lhc* culture and in those treated with exogenous Put (*wt-lhc*+Put) or Spm (*wt-lhc*+Spm). The most important result is that the thylakoidal Put/Spm ratio slightly increased (not statistically significant) after 3h of

UVB irradiation (Fig. 23). This is an inverse response as compared to that found in *wt* cultures where UVB treatment induced the reduction of Put/Spm ratio.

The measurements of ROS in *wt-lhc* cultures treated or untreated with exogenous polyamine also show that UVB did not induce the generation of ROS in *wt-lhc* (Fig. 24).

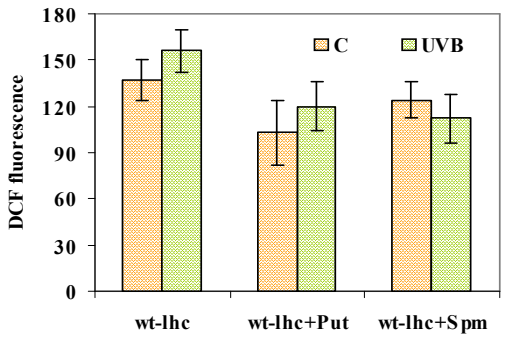


**FIGURE 24.** ROS generation in *wt-lhc* cultures supplemented or not with exogenous polyamines, as determined using a method based on the determination of DCF fluorescence, prior (C) and after 3h UVB irradiation (UVB)

The investigation of Car in *wt-lhc* cultures treated with polyamines showed that excepting lutein the other carotenoids synthesis was inhibited upon polyamine addition (Fig. 25A). Similar results were obtained for the xanthophyll pool (Vx+Ax+Zx), as well as for Vx cycle (Fig. 25B).

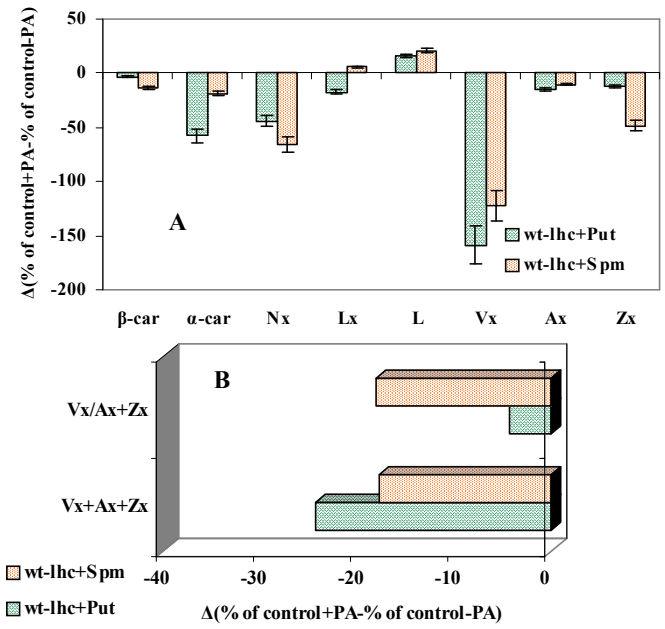


**FIGURE 25.** The influence of Put and Spm on (**A**) the pattern of carotenoids and (**B**) the β-xanthophyll cycle upon UVB exposure. The values on Y-axis represent the difference (Δ) between the values obtained for the culture treated with polyamine and those obtained for not-treated cultures, both expressed as % of control values (as determined prior to irradiation). The xanthophyll cycle is estimated as changes in the xanthophyll pool (Vx+Ax+Zx) and Vx conversion into Ax and Zx (Vx/Ax+Zx), both expressed as Δ (as described above).

## *DISCUSSION*

Under stress conditions, the potential for an energy imbalance between photochemistry, electron transport and metabolism is exacerbated and PSII excitation pressure increases (Strid et al., 1996; Karpinski et al., 1999). Several mechanisms have evolved to ensure a balance between light energy absorbed versus energy utilized through electron transport and metabolism to protect PSII from over-excitation. The most important are the dissipation of excess energy as heat and the stimulation of the capacity to utilize ATP and NADPH through metabolism to maintain high photochemical quenching of PSII excitation.

As previously showed, UVB radiation induces an increased excitation pressure on PSII. The photosynthetic apparatus reacts by increasing the size of the functional antenna to assure the dissipation of excess energy and to maintain some equilibrium between energy absorption and usage. Two mechanisms contribute to the increase in the antenna size: a) the oligomerization of LHCII and b) the transformation of active reaction centers in dissipative sinks for the excitation energy. The longer the UVB radiation treatment is the antenna size and the rate of energy dissipation increased proportionally.

An increase in the LHCII size is associated with a decrease in the Put/Spm ratio in thylakoids. In fact, the LHCII oligomeric/monomeric ratio and Put/Spm in thylakoids are changed concomitantly but in opposite directions. This relationship is much more stressed by the fact that in the absence of LHCII (as in the mutant *wt-lhc* photosynthetic apparatus), the Put/Spm ratio in thylakoids remain almost unchanged. The overall picture of responses induced by UVB radiation in the photosynthetic apparatus simulates the adaptation to LL intensities.

The conclusion drawn from the preceding data (*see* Chapters I-II) was that Put/Spm ratio in thylakoids regulates the LHCII size and, through this, the sensitivity of the photosynthetic apparatus to UVB. Based on this assumption, it was hypothesized that by manipulating the Put/Spm ratio in thylakoids, the sensitivity of the photosynthetic apparatus to UVB could be changed. Concretely, by increasing the Put/Spm ratio, the tolerance to UVB radiation can be increased or, *vice-versa*, by decreasing the Put/Spm ratio, the degree of sensitivity to UVB is increased. For this purpose, the thylakoidal titers of Put and Spm were changed by addition of exogenous polyamines. The obtained data reveal that polyamines regulate the sensitivity/tolerance to UVB by structural

readjustments of the photosynthetic apparatus (specifically, the LHCII size and composition) as it is further discussed below.

1. Spm regulates the photosynthetic apparatus sensitivity to UVB radiation through non-photochemical quenching of the absorbed light energy

The preceding results showed that HL intensities antagonize the UVB effects, determining an increase in the tolerance of the photosynthetic apparatus to UVB. The supplementation of HL-adapted cultures with Spm makes them more sensitive to UVB damage. The main cause is the increase in the oligomerization of LHCII. As in the case of exposure to LL-intensities, where the UVB effect is amplified due to the synergistic contribution of LL to the increase of LHCII size, Spm in fact induces a LL-simulated photosynthetic apparatus that exhibits more susceptibility to UVB damage.

The first evidence for the regulatory role of Spm was obtained from the investigation of biochemical changes induced after UVB treatment in the LHCII structure. The quantification of total protein amount in the LHCII sub-complexes (Fig. 12A-B) showed that Spm induces an increase in the oligomerization status of LHCII, as expressed by a higher oligomeric/monomeric fraction ratio (Fig. 12C). Examining the pattern of polyamines in the thylakoid membranes (Fig. 9) and in isolated LHCII oligomers and monomers (Fig. 11), it was found that Spm treatment leads to the reduction of Put/Spm ratio at both thylakoid and LHCII levels (Figs. 10B, 11). This finding demonstrates that the decrease in the Put/Spm ratio results from the Spm binding to thylakoids (Fig. 10A) and LHCII oligomers (Fig. 11), on the one side, and that the observed increase in the LHCII size is due to the increase in the Spm-associated to thylakoid membranes and LHCII-oligomers. The binding of Spm to the LHCII is supported by several findings. First, it was observed that Spm is efficiently conjugated to LHCII apoproteins (Serafini-Fracassini et al., 1988). Second, it was demonstrated the existence of light-stimulated transglutaminase (Dondini et al., 2003), which catalyses polyamine conjugation to the apoproteins of PSII antenna complexes (LHCII, CP24, CP26 and CP29 (Del Duca et al., 1994). Third, an Lhcb apoprotein as preferential substrate for the transglutaminase-mediated binding of Spm was recently found. Furthermore, it was suggested that polyamines can also bind chlorophylls and carotenoids, mainly chl b (Della Mea et al., 2004) and several suppositions about the

possible sites for Spm conjugation in the LHCII structure (Kühlbrandt et al., 1994) were made.

The increase in the size of LHCII by Spm was followed by a reaction cascade that constitutes the response of the photosynthetic apparatus to UVB which, as previously described, mimics low light photoadaptation. Specifically, the increase in the LHCII size (Fig. 12) results in the amplification of overexcitation induced by UVB at reaction centers and the blockage of the electron transport after $Q_A^-$ (Fig. 3C); therefore, the amount of $Q_B$ non reducing centers is increased by Spm treatment (Fig. 7C). Due to these perturbations in the function of PSII, the photochemistry (expressed by qPQ; Fig. 3D) decreases and the maximum (Fv/Fm) and operational ($\Phi_{SPSII}$) quantum yields lower (Figs. 2A, 4C). In the literature, it is reported that the down-regulation of PSII maximum quantum yield (Fv/Fm) is caused by the photochemical damage of PS II (Jansen et al., 1998; Krause et al., 1999; Rajagopal et al., 2000). In addition, protein degradation driven by UVB radiation was found to attend this loss in PSII reaction centers functionality (Friso et al., 1994). Upon UVB, the efficiency of exciton trapping is not changed by Spm (TRo/RC; Fig. 3B). Instead, the reaction centers are transformed to dissipative sinks (Tevini et al., 1991). This suggests the existence of the so-called "cruise control", a mechanism that assures a relative stable rate of excitation by increasing the dissipation of excess energy (Gruszecki et al., 1995). There is a linear correlation between the decrease in the PSII photochemistry and the inactivation of reaction centers (Fig. 2A-B), which is regulated by Spm through the increase in the functional antenna. As many reaction centers are inactivated, the functional antenna size grows proportionally (ABS/RC; Fig. 2C). The advantage of a bigger antenna is that it dissipates more excess excitation energy (DIo/RC; Fig. 2D). This photoinhibitory quenching has a protective role for the active reaction centers according to Oquist et al. (1992) and it becomes stronger as the stress is prolonged (Chow et al., 2005). Due to the PSII heterogeneity, UVB preferentially inactivates the PSII-α, whilst PSII-β reaction centers are less affected (Fig. 7). This suggests that an increase of LHCII leads to a higher susceptibility of the centers having a bigger LHCII for inactivation by UVB and raises the question if the decrease of Put/Spm ratio in thylakoids (which leads to the LHCII increasing) is a benefit for the photosynthetic apparatus or not. The final result of the alterations induced in the structure and function of the photosynthetic apparatus by Spm is the reduction of oxygen evolution (Fig. 8).

An increase in the LHCII size is accompanied by a decrease in the Chl a/b ratio (Fig. 13A), a fact that is in agreement with the data obtained from the quantification of LHCII oligomers and monomers (Fig. 12). In addition, it constitutes evidence that Spm induces a low light simulated behavior, since it is well-known that adaptation to low light intensities requires readjustments in the Chl antenna size (Masi and Melis, 1997; Navakoudis et al., 2006). By increasing the functional antenna and through this the sensitivity to UVB, it seems that Spm does not protect the photosynthetic apparatus from UVB harmful effects. In fact, the increase in the LHCII might be induced by the need for enhanced photoprotection, since a bigger antenna dissipates more excitation energy (DIo/RC, Fig. 2D) thus reducing the increased excitation pressure exerted on PSII by UVB (Fig. 4A). This finding is in agreement with previous studies reporting the involvement of LHCII trimers in the increase in the level of energy dissipation that gives rise to the non-photochemical quenching of chlorophyll fluorescence (NPQ) and which provides protection against photodamage (Chow et al., 2005; Horton et al., 1996). Additionally, it was demonstrated that the amount of LHCII proteins increases by interrupting the electron transfer from $Q_A$ to $Q_B$ with DCMU and is repressed by inhibiting the oxidation of plastoquinol with DBMIB (Escoubas et al., 1995), thus expressing the enhanced need for photoprotection.

Although the inactivation of reaction centers is higher with Spm, the investigation of *wt*-*lhc* behavior to UVB indicates that the increase in the LHCII has a protective role (Fig. 16). In both *wt* and *wt*-*lhc* cultures treated with Spm, the inactivation of reaction centers is higher than in the corresponding *wt* or *wt*-*lhc* cultures treated with Put (Figs. 2B, 17B) and this contributes to higher rates of energy dissipation (Figs. 2D, 17C) in parallel to a higher reduction in the maximal net photosynthetic rate (Figs. 8, 22). However, in the *wt*-*lhc*+Spm+UVB culture, there are no significant differences in the response to UVB radiation as compared to the *wt*-*lhc*+UVB culture. An interesting fact is that, in spite of the supplementation of *wt*-*lhc* cultures with Spm, this does not contribute to a significant reduction of Put/Spm ratio in thylakoids upon UVB (Fig. 23), although there is an evident increase in the intracellular Spm amount (Fig. 24). This suggests that Spm is mostly bound to the LHCII sub-complexes. In the *wt*+HL+Spm+UVB and *wt*-*lhc*+Spm+UVB cultures, the DIo/RC increases in parallel to the increase of functional antenna size, expressed as ABS/RC (Figs. 2C, 17C). Whilst in the former the increase in the functional antenna size results

from both LHCII increasing (Fig. 12) and inactivated reaction centers (Fig. 2B), in the last the increase in the values obtained for ABS/RC results exclusively from the inactivated reaction centers (Fig. 17B-C), as previously shown (Strasser, 1978). The amount of $Q_B$ non-reducing centers found in *wt-lhc*+Spm+UVB culture is slightly increased (Fig. 18), and equivalent to the rate of electron transport reduction (Fig. 19), demonstrating that, as in *wt*, the inactivation of reaction centers is due to the blockage of electron transport to $Q_B$. In addition, the energetic connectivity increased in *wt*+ HL+Spm+UVB culture (Fig. 3A) whilst in *wt-lhc*+Spm+UVB is maintained close to control (data not shown).

Although the Chl biosynthesis in *wt* is not significantly affected by Spm addition, there is an increase in the total Car amount, due to the stimulation of lutein accumulation, as well as, Lx and Nx (Fig. 13B). This is probably related to the changes in the oligomerization status of LHCII. Lutein is important for the stabilization of LHCII sub complexes (Bishop, 1996), and its increase is needed to achieve a bigger antenna with a higher capacity of energy dissipation (high-quenched antenna status). In both *wt* and *wt-lhc* cultures, Spm also does not stimulate the xanthophyll cycle (Figs. 13C, 26B), a result that is in agreement with the previously reported finding that UVB inhibits Vx de-epoxidation (Pfündel et al., 1992). However, all the data obtained for the β-xanthophyll cycle (the conversion of Vx into Ax and Zx) in both *wt* and *wt-lhc* cultures showed that Vx cycle is not operating under UVB exposure. Thus, the higher rate of thermal dissipation is not related to the quenching of Chl a* molecules by zeaxanthin as it was previously showed (Horton et al., 1996). In fact, Spm influences, to a much smaller degree, the structure and function of the photosynthetic apparatus in the *wt-lhc* mutant than in the *wt* culture (Figs. 6, 21). This suggests that, even at the control level, the regulation of the structure and function of the photosynthetic apparatus by Spm during UVB stress requires the presence of LHCII.

The oligomerization of LHCII by Spm makes the photosynthetic apparatus more sensitive to UVB. In this context the question should be asked why the photosynthetic apparatus chooses to respond to UVB stress by increasing Spm in thylakoids, and, subsequently, its LHCII antenna and not inversely, since Put seems to antagonizes the UVB effect conferring more protection to UVB ?

Recently published data demonstrates that in several stress conditions (i. e. chilling (Sfakianakis et al, 2006), enhanced UVB radiation (Sfichi et al., 2004; Lütz et

al., 2005), enhanced tropospheric ozone concentration (Navakoudis et al., 2003) the photosynthetic apparatus reacts in the same manner, namely lowering the Put/Spm ratio and increasing the LHCII antenna size. In all the above situations, the stressor induced the overexcitation of PSII that causes the downregulation of the photosynthetic capacity. Previously, it was affirmed that there are two ways to decrease the excitation pressure: by increasing the photochemistry and by increasing the non-photochemical quenching (Huner et al, 1998). The photochemistry increase requires certain conditions, such as adjustments in the functional antenna size which will reduce the amount of light energy trapped at reaction centers or an increase in the number of components acting as electron-consuming sinks by elevating the levels of Calvin cycle enzymes, which would increase the capacity for $CO_2$ assimilation or photorespiration relative to electron transport. All these responses can be developed on a longer time scale (hours to days). Thus, the other alternative is to increase the non-photochemical quenching potential, which can be done on a time scale of minutes (Huner et al., 1998; Ruban et al., 2005; Ioannidis et al., 2006; Ioannidis PhD, 2006).

It seems that during UVB stress, the latter way is preferred as a means to resist against the high excitation pressure exerted on PSII. In order to initiate the reactions that will assure resistance to UVB damage and recovery through enhancing non-photochemical quenching, Spm is acting as primary response of the photosynthetic apparatus to UVB. Besides its role in determining the oligomerization of LHCII, Spm increases the connectivity between the photosynthetic units (Fig. 3A). These two phenomena regulated by Spm may ensure the recovery of the photosynthetic apparatus structure and function after the cessation of UVB radiation. The fact that in *wt-lhc*+Spm+UVB cultures there is no recovery although the damage was not as strong as in *wt*+HL+Spm+UVB cultures may constitute an argument for the above hypothesis. High Spm levels lead to structural re-adjustements ("umbrella") that contribute to the thermal dissipation of the excess energy and also protect the reaction centers from irreversible damage (*see* Chapter II). First candidates to participate in the creation of such structure are the PSII-α reaction centers (RCs) which possess a higher antenna and assure the functionality of the photosynthetic apparatus under physiological conditions. When these PSII-α RCs are inactivated by the stressor agent and act as dissipative sinks for the excitation energy, the functionality of the photosynthetic apparatus is assured by PSII-β RCs (Figs. 28-29). The intrinsic trapping and fluorescence properties of PSII-α and PSII-

β RCs are considered to be similar. However, some studies reported distinct action spectra and emission spectra, showing that PSII-α centers are richer in chlorophyll b than the PSII-β centers. Most of the authors identify the $Q_B$ non-reducing centers with a fraction of PSII-β and some of them consider these centers as a minor component of PSII-α connected with the active centers (Lazár et al., 2003). After the cessation of UVB stress action, the recovery mechanisms assure the repair of the photosynthetic units. Consequently, Spm plays the role of binding agent of the photosynthetic units.

Overall, the mechanism regulated by Spm needs the presence of LHCII, which in the oligomerization state assures a high dissipation of excess energy and contributes to readjustments in thylakoids membranes that lead to the formation of the dissipative supercomplex. Oligomerization of LHCII may therefore be important in increasing the stability of thylakoid membranes, since it is well-known that is essential for the grana stacking (Chow et al., 2005). In addition to the above effects, Spm could participate directly in the dissipation of excess excitation energy. Ioannidis (unpublished data) demonstrated that an increase of energy dissipation seems to be a direct effect of Spm on the LHCII and indicated the pair Spm-Chl as minimal structural unit of NPQ. It is likely that Spm binds preferentially the Chl b molecules (Della Mea et al., 2004). According to current concepts, the fluorescence quenching could be due to LHC II aggregation, carotenoid cation formation or Chl excimer formation (Horton et al., 1991; Wenthworth et al., 2004; Robert et al., 2004; Ruban et al., 2005). By means of Chl b and conjugation of LHCII apoproteins, it is possible that Spm connects the photosynthetic units between them (Fig. 27). In conclusion, the sensitivity to UVB radiation and its capacity for recovery is determined by the size and composition of LHCII, which is regulated by the Spm-associated to thylakoids (and LHCII oligomeric forms). This seems to be the primary response of the photosynthetic apparatus to UVB. The fact that exogenous Spm by increasing the LHCII antenna size accentuates the sensitivity of the photosynthetic apparatus to UVB (as it appears in HL conditions) is the strongest evidence for the above conclusion.

2. Put increases the photosynthetic apparatus tolerance to UVB radiation by enhancing the photochemistry

The externally supplied Put was proven to enter the cells raising its intracellular level without conferring any toxic affects or photoinhibition (Fig. 6), a fact that is

consistent with previously reported data (Govindachary et al., 2004; Navakoudi et al., 2006). The increase in the Put/Spm ratio in thylakoids (Figs. 9, 10B) and LHCII oligomers (Fig. 11) after supplemental addition of exogenous Put in LL cultures, resulted in an increase of the Fv/Fm ratio (Fig. 2A), due to inhibition of UVB quenching effect on Fm (Fig. 1). Put inhibits the increase in the oligomerization status of LHCII, expressed as oligomeric/monomeric ratio (Fig. 12C). By its action, Put induces HL-photoadaptive responses in the photosynthetic apparatus that antagonizes the effect of UVB and reduces the damage. Consequently, the photosynthetic apparatus becomes more tolerant to UVB. This finding is consistent with data reported by other investigators that Put induces HL-adaptive responses (Anderson et al., 1988, Chow et al., 2005; Papadakis et al., 2005; Navakoudi et al., 2006).

The effect of Put on LHCII size seems to be indirectly determined by Put as a consequence of Put-induced stimulation of photochemical reactions. In the presence of Put, more reaction centers are kept active (Fig. 2B), and, subsequently, this assures higher electron transport rates (Fig. 3C) and an increase in the pool of oxidized PQ (Fig. 3D). According to Strasser et al. (1995), the increase in the number of PSII reaction centers may be a reflection of the increased electron transport chain capacity. To increase the number of PSII reaction centers and at the same time to maintain a balance of excitation energy between the two photosystems requires a decrease in the LHCII antenna size. As a result of a decrease in LHCII size and an increase of active reaction centers density, the functional antenna size (ABS/RC) is also decreased by Put and, subsequently, a lower amount of excess energy is dissipated upon UVB stress (Fig. 2C-D). Consequently, there is an increase in the photochemical quenching capacity of the photosynthetic apparatus leading to the enhancement of oxygen evolution (Fig. 8). Increasing the PQ pool (Fig. 3D), the electron transport becomes more efficient (Fig. 3C) and the maximum and operational quantum yield efficiencies also increases (Figs. 2A, 4C). The rapid cycle of oxidation/reduction of PQ (as denoted by qPQ) diminishes the amount of $Q_B$ non-reducing centers (Fig. 7C). This enhancement of the photosynthetic activity by Put is explained by the fact that Put can stimulate ATP synthesis. It was recently demonstrated that a concentration of Put of 1-2 mM increase the chemiosmotic ATP synthesis (without any changes in the light conditions) more than 70% (Ioannidis et al., 2006). By stimulating the ATP synthesis, Put diminishes the amount of dissipated energy (Fig. 2D). This finding is in accordance with the recent proposal that *in vivo*

modulation of NPQ is regulated through the chloroplast ATP synthase and stromal metabolites (Kanazawa and Kramer, 2002; Ioannidis et al., 2006).

Another effect of Put is the decrease in the energetic connectivity between the photosynthetic units (Fig. 3A) and therefore preferentially induced photochemical (and not the non-photochemical) quenching. At the whole cell level, no significant change was observed in *wt* after Put addition (data not shown), a fact suggesting that Put is preferentially acting on thylakoid membranes. In contrast, the mutant cultures exhibited higher polyamine content at the whole cell level (Fig. 24), supporting the fact that when the LHCII is present, Put is mobilized to thylakoids. Also, Put seems to exert an inhibitory effect on Car and Chl contents (data not shown) and this may be related to its ability to inhibit the antenna size increase. This is also consistent with previous reported data that the diminution of Put in plants exposed to UVB is concomitantly with the increase in the carotenoids synthesis (Lütz et al., 2005).

Despite its protective effect on the reaction centers, Put accumulation in thylakoid membranes isolated from *wt*-*lhc*+Put+UVB cultures (as described by an increased Put/Spm ratio) (Fig. 24), does not help the *wt*-*lhc* cultures to recover from the UVB effects even 4 h after the cessation of UVB treatment. In contrast, the *wt* photosynthetic apparatus maintains its recovery potential. This is the strongest evidence showing that, in spite of the smaller amplitude of damage, recovery is not possible without LHCII. Put protects some reaction centers counteracting the inactivation induced by UVB, but this is not sufficiently to assure recovery, which in the last instance means the repair of damaged reaction centers. Thus, LHCII is required for the restoration of the photosynthetic ability after UVB effects, even if Put assures certain protection by increasing the functionality of reaction centers. It seems that the importance of LHCII in the mechanism determining the degree of sensitivity to UVB consists not only of its capacity to keep the balance between energy captured at reaction centers and dissipation of excess energy, but also contributes to the stabilization of membranes. Since the light harvesting complex of PSII (LHCII) plays an important role in light absorption, and energy transfer to the reaction center as well as thylakoid stacking (Chow et al., 2005), any damage to these systems results in multiple effects on the photosynthetic apparatus. An interesting fact is that ROS were not found in any of the *wt* or *wt*-*lhc* investigated cultures (Figs. 14-15, 24). Thus, it cannot be supposed that the sensitivity of UVB radiation is regulated by ROS, through changes in the polyamine pattern and LHCII size.

## *Conclusions*

Overall, the main findings of the present study (*Chapter III*) are the following:

1. The excitation pressure exerted on PSII by UVB seems to be the primary signal for the modification of Put/Spm ratio in thylakoids. The data presented here lead to the conclusion that changes of thylakoid-associated polyamine act as a primary mechanism which adjust the degree of sensitivity of the photosynthetic apparatus to UVB radiation by regulating the size of the functional antenna and therefore the photochemical and non-photochemical quenching of absorbed energy without the production of AOS.

2. UVB simulates the same molecular and bioenergetical changes as in a photosynthetic apparatus adapted to low light conditions (low Put/Spm ratio in thylakoids leads to a LHCII-size increase, inactivation of reaction centers and therefore to an enhanced non-photochemical quenching). Photoadaptation to high light conditions induces exactly the opposite changes (high Put/Spm ratio in thylakoids leads to a LHCII-size decrease, activation of reaction centers and therefore to an enhanced photochemical quenching). Therefore HL-adaptation acts antagonistically to UVB effect and enhances the tolerance against UVB radiation. LL-adaptation amplifies the UVB effect and therefore minimizes the tolerance and enhances the sensitivity to UVB.

3. The excitation pressure of PSII adjusts the balance of Put and Spm level in thylakoids and especially in LHCII forms. Nature, in order to protect the photosynthetic apparatus against UVB, had to decide (during the evolution) between Put and Spm for the protection of the photosynthetic apparatus against UVB stress. In the case that nature adopted as a primary response to UVB an increase of the thylakoid-associated Put (increase of the Put/Spm ratio), that would lead to a photosynthetic apparatus phenotype with enhanced photochemical (and reduced non-photochemical) quenching, that is able to minimize the excitation pressure exerted on PSII by UVB stress. In case that nature adopted as primary response to UVB an increase in the thylakoid-

associated Spm (decrease of the Put/Spm ratio), that would lead to a photosynthetic apparatus phenotype with enhanced non-photochemical (and reduced photochemical) quenching, that again is able to minimize the excitation pressure on PSII and to resist against UVB stress. Nature prudently decided for the second mechanism, because under normal irradiance conditions, the intensity of UVB radiation increases proportionally to the increase of the visible light intensity. The adoption of the second mechanism (the decrease of Put/Spm ratio), under physiological light conditions (HL) gives to the photosynthetic apparatus an advantage for further attenuation of the UVB impact by the antagonistic effect of high (visible) light intensities.

4. Comparative experiments with *wt* and mutant (*wt-lhc*: similar to *wt* without LHCII) cultures confirm that the size and the composition (oligomerization/monomerization) of LHCII regulate the sensitivity/tolerance of the photosynthetic apparatus and therefore of the organism against UVB.

5. Additionally, through artificial changes of the Put/Spm ratio (exogenous supplied polyamines) it is possible to simulate LL-adapted or HL-adapted photosynthetic apparatus and therefore absolutely tolerant or sensitive organisms towards UVB, independent from the ambient light conditions.

**A model of the regulation of photosynthetic apparatus sensitivity to UVB radiation**

The data presented in this work can be comprised in a model of regulation of the photosynthetic apparatus sensitivity/tolerance to UVB radiation (Fig. 26). According to this, UVB radiation increases the excitation pressure on PSII, which regulates the levels of Put and Spm in thylakoids in function of the adaptation degree of the photosynthetic apparatus to PAR intensity conditions. In low PAR conditions (LL-adapted plants) the sensitivity of the photosynthetic apparatus to UVB is high. There is an increase in the Spm amount (low Put/Spm ratio in thylakoids) which induces structural re-adjustments that assure the dissipation of excess energy through non-photochemical quenching. Specifically, an increase in Spm leads to the oligomerization of LHCII followed by an increase in the inactivation of reaction centers and, subsequently, of the photochemistry rate. In turn, LHCII and inactivated reaction centers contribute to the building of a

structure characterized by high non-photochemical quenching capacity. This structure also assures the restoration of the initial status of the photosynthetic apparatus after UVB stress. The fact that a *wt-lhc* mutant has no capacity to recover the changes induced by UVB in the photosynthetic apparatus demonstrates that LHCII prevents the irreversible damage. The primary photoreceptors responsible for the photosynthetic apparatus sensitivity to UVB are chlorophylls (656/430 nm), which increase the excitation pressure exerted on PSII by UVB.

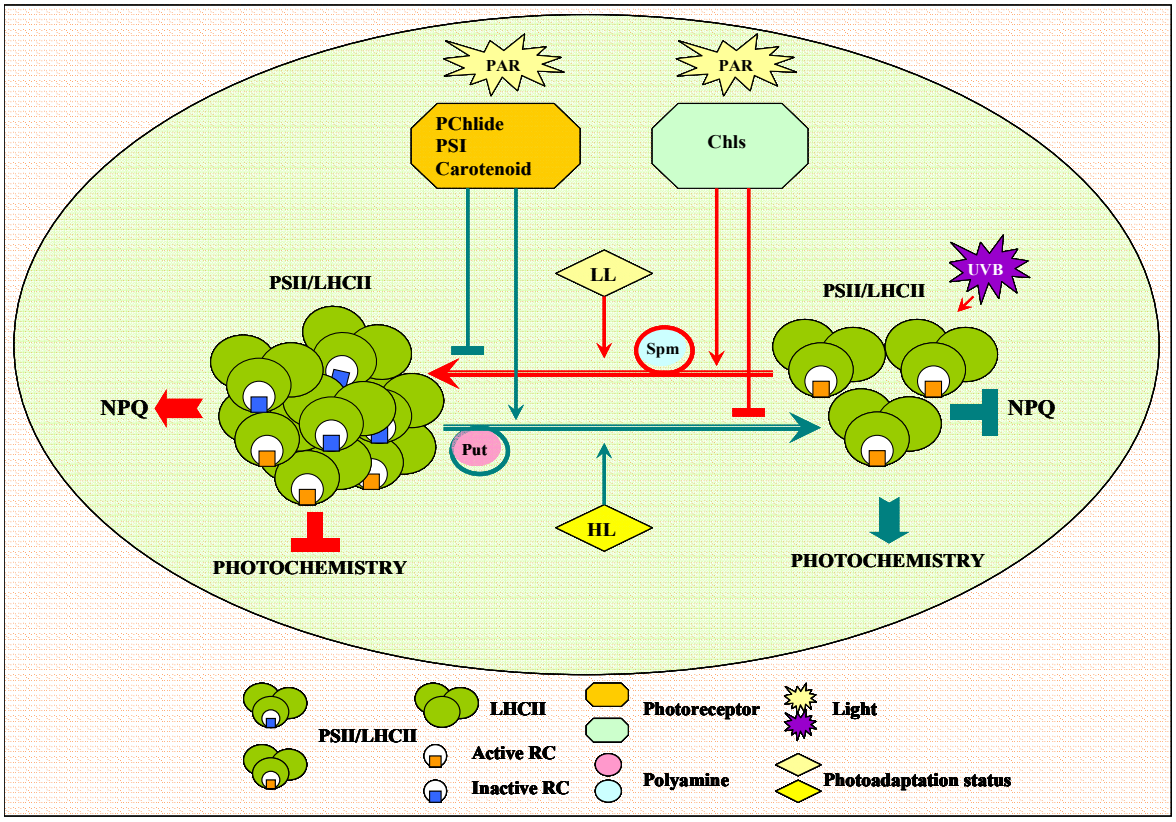


**FIGURE 26.** Model for the regulation of the photosynthetic apparatus sensitivity/tolerance to UVB radiation (for details, *see* text)

In high PAR intensity conditions (HL-adapted plants) the photosynthetic apparatus is more tolerant to UVB stress. Due to an enhanced photochemistry, the excitation pressure exerted on PSII by UVB is minimized. High photosynthetic rates require Put (high Put/Spm ratio in thylakoids) which it was shown to stimulate the chemisomotic ATP synthesis (Ioannidis et al., 2006). As a result, there is an increase in the density of active reaction centers whilst the LHCII is kept low, in order to minimize the loss of the excitation energy through non-photochemical quenching mechanisms. The

primary photoreceptors responsible for the tolerance of the photosynthetic apparatus to UVB are PChlides (620-640/442 nm), PSI (690-730 nm) and a carotenoid absorbing at 535 nm. They contribute to maintain low the excitation pressure exerted on PSII by UVB.

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