REGULATION OF PHOTOSYNTHESIS AND ENERGY DISSIPATION IN
TRIAZINE-RESISTANT AND SUSCEPTIBLE *CHENOPODIUM ALBUM*

REGULATIE VAN FOTOSYNTHESE EN ENERGIEDISSIPATIE IN
TRIAZINE-RESISTENTE EN GEVOELIGE *CHENOPODIUM ALBUM*

Victor Bas Curwiel

# REGULATION OF PHOTOSYNTHESIS AND ENERGY DISSIPATION IN TRIAZINE-RESISTANT AND SUSCEPTIBLE *CHENOPODIUM ALBUM*

Cover design: Victor B. Curwiel and Allex B. Haasdijk

# Stellingen

1. Onderzoek bij *Chenopodium album* naar het effect van abiotische stress op triazine-resistente planten heeft aangetoond, dat stress kan leiden tot schaduwtype eigenschappen.

   Dit proefschrift.

2. Het verschil in de gevoeligheid van triazine-resistente en "wild-type" planten van *Chenopodium album* voor fotoinhibitie komt het sterkst tot uiting bij hoge lichtintensiteit tijdens de opkweek.

   Dit proefschrift.

3. Van alle mechanismen, die *Chenopodium album* ter beschikking heeft om zich te beschermen tegen overdadig licht zijn die, welke leiden tot fotochemische (qP) en energie-gerelateerde (qE) doving van chlorofyl *a* fluorescentie, het belangrijkste.

   Dit proefschrift.

4. De aanwezigheid van grote hoeveelheden van het xanthofyl pigment zeaxanthine in en om de nabijheid van de pigment-eiwit complexen van het 'light harvesting complex' van fotosysteem II hoeft geen rechtstreeks effect te hebben op de capaciteit van de fotobeschermende energie dissipatie.

   Dit proefschrift.
   Hurry *et al.*, 1997. Plant Physiol. **113**, 639-648.

5. Bij de aanschaf van een nieuwe personal computer willen de gebruikers steeds een snellere, grotere en duurdere machine, terwijl er tegelijkertijd steeds minder gebruik gemaakt wordt van zijn potentiële mogelijkheden.

6. Alhoewel film door de jaren heen een uitstekend cultureel medium is gebleken om een boodschap aan het publiek over te brengen, wordt de boodschap niet altijd even goed begrepen.

7. Het schrijven van het proefschrift is voor de promovendus de beproefde methode om alle feiten logisch op een rijtje te zetten.

8. Het uitdelen van oscars aan genomineerde films blijft een interne Amerikaanse aangelegenheid, terwijl het toekennen van prijzen aan werkelijk goede films alleen is voorbehouden aan de Europese filmfestivals (Berlijn, Brussel, Cannes, Parijs, Rotterdam, Venetië).

9. De hype Internet is niets anders dan een aaneenschakeling van computerservers, zonder kop en staart, waar permanent een chaos heerst.

10. Milieuactivisten zijn bewogen met het wel en wee van de natuur, maar deze bewogenheid wordt niet in alle gevallen op een logische wijze ten toon gespreid.

11. Sporten is goed voor geest en lichaam, maar elke sporter is en blijft een gezonde invalide.

12. Het schrijven van artikelen blijft voor de meeste wetenschappers een kwelling van geest en lichaam wanneer de auteurs van mening blijven verschillen met de reviewers van het manuscript.

Stellingen behorend bij het proefschrift "Regulation of photosynthesis and energy dissipation in triazine-resistant and susceptible *Chenopodium album*".

Wageningen, 26 juni 1997                                    Victor Bas Curwiel

# Voorwoord

Toen ik na het beëindigen van mijn studie landbouwplantenteelt aan de Landbouwuniversiteit te Wageningen de mogelijkheid kreeg aangeboden om bij de (toenmalige) vakgroep Plantenfysiologisch Onderzoek gedurende een jaar op vrijwillige basis onderzoek te doen op het gebied van herbicide-resistentie en fotosynthese, greep ik deze kans met beide handen aan om mijn interesse en ervaring op dit vlak verder uit te breiden. Het gebruik van resistente planten om het effect van stress op planten te bestuderen had al tijdens mijn eerste afstudeervak op de vakgroep Plantenfysiologisch Onderzoek mijn enthousiasme en interesse voor dit onderzoek aangewakkerd. Aansluitend op het NOP (Na-doctoraal Onderzoek Projekt) "Licht-geïnduceerde proton translocaties in geïsoleerde chloroplasten" ben ik begonnen met het AIO-projekt "Invloed van herbicide-resistentie en abiotische stress op de fotosynthese".

Al gedurende mijn eerste afstudeervak en tijdens het NOP-projekt werd ik begeleid door mijn co-promotor Jacques J.S. van Rensen. Ook tijdens mijn AIO-schap was Jacques mijn steun en toeverlaat tijdens mijn vele proeven en onderzoek op de vakgroep Plantenfysiologie. De vele discussies en besprekingen van het onderzoek hebben mij doen beseffen dat jouw hulp en kundigheid nodig zijn geweest om dit promotieprojekt tot een goed einde te brengen. Al voor het AIO-schap had je me rondgeleid in het internationale veld tijdens het 8e FESPP congres in Antwerpen waarbij je me alle facetten van deze onderzoekswereld liet zien. Dankzij een goed samenwerkingsverband hebben we samen de nodige artikelen kunnen schrijven. Ook mijn promotor Wim Vredenberg ben ik dankbaar voor zijn inzet tijdens de bespreking van het proefschrift. Ik denk dat het heel nuttig is geweest dat je als fysicus een heel andere frisse kijk had op de door mij uitgevoerde experimenten. De met je gevoerde discussies zijn ten goede gekomen aan de kwaliteit van het proefschrift.

De studenten, die ik tijdens mijn AIO-schap bij hun afstudeervak heb begeleid ben ik allen dankbaar voor hun werk en inzet. Met name Anne-Marie Derksen en Linda de Poorter hebben met hun experimenten enige openstaande vragen van mijn onderzoek kunnen beantwoorden en daar ben ik hun zeer dankbaar voor. Voor het kweken van de nodige planten voor mijn proeven gaat mijn dank uit naar het kaspersoneel en met name naar Leen, die de afgelopen jaren onafgebroken veel moeite gedaan heeft om uitstekend plantenmateriaal te

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Voor de meeste onderzoekers blijft de AIO- of OIO-periode de leukste en meeste intrigerende fase uit hun onderzoeksleven en ook voor mij waren de buitenlandse reizen zowel leerzaam als amusant. Ze gaven me de kans om collega's te ontmoeten en via e-mail en bezochte summer schools heb ik een leuke kennissenkring opgebouwd.

Verder gaat mijn dank uit naar alle andere collega's, die me tijdens mijn verblijf op de vakgroep in zekere mate hebben geamuseerd en ondersteund. Hans, ik zou zeggen blijf lachen en *keep yourself in shape*. Ria wil ik bedanken voor haar morele steun bij het afronden van dit proefschrift en Paul veel succes met je verdere loopbaan en hou de eer van FOTS hoog in het vaandel! *Last but not least, Rose thanks for proof-reading my thesis* en ook Marcel bedank ik voor het zorgvuldig nakijken van mijn proefschrift.

Tenslotte wil ik dit voorwoord eindigen met een dankwoord gericht aan mijn ouders, die niet alleen tijdens mijn AIO-schap, maar ook tijdens mijn gehele studieloopbaan me altijd hebben gesteund en gemotiveerd om door te studeren. De promotie is daarmee tevens de afronding van een zeer lange en geslaagde studie. Pa en Ma bedankt voor alles!

# Contents

# Abbreviations

| | |
|---|---|
| APO | ascorbate peroxidase complex |
| atrazine | 2-chloro-4(ethylamino)-6-(isopropyl-amino)-*s*-triazine |
| CCCP | carbonylcyanide-*m*-chlorophenylhydrazone |
| Chl | chlorophyll |
| D1 protein | Photosystem II reaction center protein containing the $Q_B$-binding site |
| DAD | diaminodurene |
| DCPIP | 2,6-dichlorophenolindophenol |
| DTT | dithiotreithol |
| ET | electron transport |
| FeCy | potassium hexacyanoferrate(III) |
| Fm | Chl *a* fluorescence level when all Photosystem II reaction centers are closed |
| Fo | Chl *a* fluorescence level when all Photosystem II reaction centers are open in dark-adapted state |
| Fv | variable fluorescence; Fm-Fo |
| Fo' | minimum fluorescence level when all Photosystem II reaction centers are open in light-adapted dark state |
| Fm' | yield of chl *a* fluorescence due to a saturating light pulse under steady state light conditions |
| F | steady state fluorescence level in the light |
| Fv/Fm | a measure for the efficiency of open reaction centers used to determine the level of photoinhibitory damage |
| HI | high irradiance |
| J | PSII dependent electron transport |
| LHCII | light harvesting complex of PSII |
| LI | (extreme) low irradiance |
| MV | methyl viologen |
| NaMo | sodium molybdate |
| PAR | photosynthetic active radiation (400 - 700 nm) |
| PI | photoinhibition |
| PIT | photoinhibitory treatment |
| PMS | phenazine methosulphate |

| PP | photophosphorylation |
|---|---|
| PSI | photosystem I |
| PSII | photosystem II |
| $Q_A$ | primary quinone electron acceptor of PSII |
| $Q_B$ | secondary quinone electron acceptor of PSII |
| qE | high energy state dependent chlorophyll $a$ fluorescence quenching |
| qI | photoinhibition dependent Chl $a$ fluorescence quenching |
| qN | non-photochemical Chl $a$ quenching |
| q0 | quenching of Fo |
| qP | photochemical quenching of Chl $a$ fluorescence |
| qT | state transition dependent Chl $a$ fluorescence quenching |
| R | triazine-resistant biotype |
| RFC | relative fluorescence change |
| S | triazine-susceptible biotype |
| $SV_N$ | Stern-Volmer coefficient of the non-photochemical quenching |
| V | violaxanthin |
| VDE | violaxanthin de-epoxidase |
| $\Delta pH$ | trans-thylakoid proton gradient |
| $\Delta S_{535}$ | relative absorbance change at 535 nm (change in light scattering) |
| $\Delta Z$ | the total amount of light-induced zeaxanthin formation determined by relative absorbance changes at 505 nm and 535 nm |
| $\phi_P$ | photochemical quantum yield of PSII electron transport |

# CHAPTER 1

# GENERAL INTRODUCTION

## Photosynthesis

Photosynthesis is the process in higher plants and green algae that produces chemical energy (ATP) and reductive power (NADPH) by utilising light energy, water and carbon dioxide ($CO_2$). The process involves the transfer of electrons through photosystems II and I of the thylakoid membrane in the chloroplasts, which leads to charge separation across the membrane. Concomitantly with this electron transport, a proton gradient ($\Delta pH$) is built up which together with the trans-thylakoid electrical potential difference forms the proton motive force ($\Delta \mu_H^+$) which induces the production of ATP through the process of photophosphorylation via the enzyme ATPase.

qT mechanism

qE mechanism    qI mechanism

Sun light    PSII    PHOTOSYNTHESIS
(electron transport)

Heat    Photoinhibition

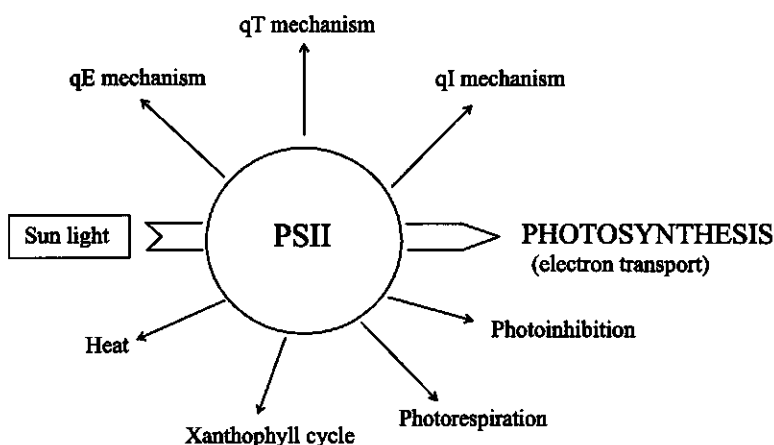Xanthophyll cycle    Photorespiration

Fig. 1.1. Scheme illustrating pathways of energy conversion in photosystem PSII.

Abiotic stress factors, *e.g.* high and low temperature, drought, high irradiance, air and soil pollution, can considerably reduce crop yields. The effect of these environmental stress factors on photosynthesis, which forms the main source of dry matter production, has been studied for many years. Higher plants are very flexible in adapting to changes in their environment, *e.g.* light irradiance, temperature, humidity. These plants can close their stomata to avoid excessive transpiration, change their leaf position to decrease/increase light absorption, move their chloroplasts (Park *et al.*, 1996b) and alter their leaf composition (lipids, sugars) to withstand cold or heat (Bender *et al.*, 1992; Fuks *et al.*, 1992). Compared to animals, plants are not able to move to shaded area shelter by sitting in the shade in case of

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excessive irradiance during day time. If a change of leaf position can not prevent the plant from receiving an excessive amount of light energy that cannot all be converted by photochemistry (photosynthesis), the plant has to take other measures to avoid possible damage to the photosynthetic apparatus (Fig. 1.1).

Most of these mechanisms aim to dissipate light energy before it can harm the plant. Such mechanisms are active at a much shorter timescale compared to long-term adaptation to high irradiance which involves a change in the balance between the synthesis and degradation of proteins and pigments (Dau, 1994). Additional environmental stresses, such as temperature and water stress, lower the photosynthetic rate and can enlarge the degree to which absorbed light becomes excessive, increasing the need for energy dissipation (Demmig-Adams and Adams, 1996). Earlier research has clearly shown that plants, next to changing the photosynthetic capacity, activity of energy dissipative pathways and pigment-protein composition, can also alter the morphological structure of their leaves (Louwerse and Zweerde, 1977).

When protective mechanisms reach saturation, the plant will be damaged. The damage by excessive light is called photoinhibition. Some optical parameters and/or effects associated with the energy dissipative pathways will be discussed in more detail in the following paragraphs: fluorescence, xanthophyll cycle and photoinhibition.

Sometimes stress is induced intentionally, using abiotic factors. For instance herbicides are used for weed control. Mechanisms of herbicide action have been studied extensively. The question whether or not a stress factor like herbicides shows synergism with the aforementioned environmental stress factors has become relevant and gained attention. This work deals with the effect of abiotic stress factors in a particular plant phenotype which has developed a resistance against herbicide action, specifically the triazine-resistant phenotypes of *Chenopodium album*.


## Triazine-resistance


Triazine resistance was first noticed by Ryan (1970), who reported that atrazine and simazine no longer controlled *Senecio vulgaris* at a nursery, where triazine herbicides had been used annually for a long time. Since, resistance has spread throughout many plants all over the world, including many weeds, *e.g. Chenopodium album* and *Brassica napus*.

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Recently resistance to atrazine has been incorporated into crops via biotechnological means. For a review about triazine resistance, *c.f.* Van Rensen and De Vos (1992).

Triazine resistance is caused by a mutation in the D1 protein at the $Q_B$ binding site (Fig. 1.2). The amino acid serine is replaced by glycine, resulting in a reduced electron flow between $Q_A$ and $Q_B$ (Jansen *et al.*, 1986; Jansen and Pfister, 1990).
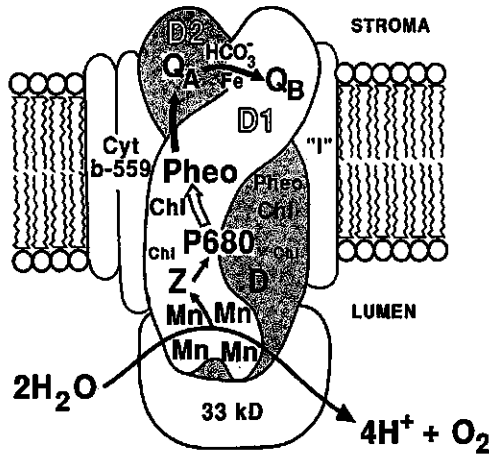


Fig. 1.2. Schematic display of photosystem II (PSII) in the thylakoid membrane of the chloroplast (Govindjee and Van Rensen, 1993).

In the resistant plants the electron transport between $Q_A$ and $Q_B$ will not be inhibited in the presence of atrazine, but in the susceptible ones atrazine blocks the electron flow between $Q_A$ and $Q_B$ leading to inhibition of photosynthesis (Naber and Van Rensen, 1991). This resistance does not cause a complete lack of binding of triazine herbicides to the D1 protein, but the residence time of these herbicides in resistant plants is about 100 times shorter (58 ms) compared to the wild susceptible biotype (6.7 s).

In the absence of triazine herbicides, the resistant plants have a lower rate of electron flow between $Q_A$ and $Q_B$ (Pfister and Arntzen, 1979; Jansen and Pfister, 1990) as compared to the susceptible plants. The impaired rate of electron transport at the acceptor side of PSII was suggested to be related to a significantly lowered rate of light- and $CO_2$-saturated photosynthesis, growth and productivity at the whole plant level (McCloskey and Holt, 1990 and references cited). However, several researchers failed to show a lowered photosynthesis rate in resistant plants (Van Oorschot and Van Leeuwen, 1984; Jansen *et al.*, 1986). This controversy

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was resolved when it was reported (Hart and Stemler, 1990a; Van Rensen *et al.*, 1990) that resistant plants, grown at low irradiance, did not differ in productivity while there was indeed a lower productivity when the plants were grown at high irradiance. It was further demonstrated that resistant plants are more sensitive to photoinhibition (Hart and Stemler, 1990b; Van Rensen *et al.*, 1990; Curwiel *et al.*, 1993).

Furthermore it was suggested that the mutation in the D1 protein not only changes kinetics of the electron flow between $Q_A$ and $Q_B$ and lower primary photosynthetic efficiency, but also affects other properties of the PSII complex important for regulation of photosynthesis and biomass production. In resistant plants the D1 protein turnover would be higher, increasing the demand for energy resources at the expense of growth and biomass production (Sundby *et al.*, 1993).

Studies of these resistant plants have revealed that their chloroplasts have shade type characteristics: different lipid composition of the thylakoid membrane, more and larger grana, more LHC associated with photosystem II, less CF1 (ATPase) and a lower chlorophyll *a/b* ratio (Burke *et al.*, 1982: Vaughn and Duke, 1984; Vaughn, 1986).

The fact that stress conditions often initially result in an impaired activity of photosystem II, makes triazine-resistant plants very suitable to be used as model plant to study the relation between abiotic stress and photosynthesis.


## Chlorophyll fluorescence


Chlorophyll fluorescence transients in a light pulse were first discovered in 1931 by H. Kautsky. Chlorophyll fluorescence has become a routine probe for information on the various aspects of photosynthesis: the identity of the various pigments and pigment complexes, their organisation, excitation energy transfer among them and on the various electron transfer reactions, specifically of PSII (Krause and Weis, 1991; Govindjee, 1995). New non-invasive fluorescence techniques have been developed to study the influence of stress on the photosynthesis process (Schreiber *et al.*, 1986; Genty *et al.*, 1989; Van Kooten and Snel, 1990).

Most of the light absorbed by the photosynthetic apparatus is converted photochemically (Fig. 1.1). Chlorophyll fluorescence is only a minor competing process of deactivation of excited pigments. When all reaction centers are open ($Q_A$ in oxidised state), the fluorescence

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yield is less than 1%. In the case of excessive radiation, all reaction centers get closed and $Q_A$ is reduced. Also, the amount of light which is re-emitted in the form of fluorescence may increase up to 3% (Krause and Weis, 1991). At room temperature, the major part of the fluorescence is emitted by PSII (LHCII). Depending on the state of the photosynthetic apparatus of the plant, more or less fluorescence is being produced. When plants are stressed (*e.g.* after chilling or after photoinhibition), the photosynthetic apparatus will function less optimal. A lower amount of light can be converted photochemically inducing a higher level of fluorescence. This makes the measurement of fluorescence a good instrument to determine the physiological state of the plant (Van Kooten and Snel, 1990).

Plants have several mechanisms by which they can convert or dissipate excessive irradiance through non-radiative energy dissipation (Krause, 1988; Horton *et al.*, 1994; Fig. 1.1). Examples of these mechanisms are heat emission and photorespiration. Photorespiration occurs in C3 plants and be described as an overflow of reductive energy which is initiated by the reaction of Rubisco with oxygen. Photorespiration can be lowered by decreasing the oxygen concentration down to 2%. The xanthophyll cycle and photoinhibition will be introduced later in this chapter. Another important indicator of photoprotective processes is fluorescence quenching.

The chlorophyll fluorescence yield can be decreased by mechanisms which are either directly or indirectly related with photochemistry, photochemical quenching (qP) and non-photochemical quenching (qN) respectively. The coefficient for photochemical quenching (qP) is related to the oxidised state of $Q_A$, more photochemistry leads to a lower qP. The qN includes three major mechanisms usually having different relaxation kinetics:

1) qE, high energy state quenching, related to the light-driven proton gradient across the thylakoid membrane and with relaxation kinetics in the one to two minute range.

2) qT, quenching related to state 1 - state 2 transitions, regulated by phosphorylation of LHCII and with relaxation kinetics in the timescale of 5 - 10 min.

3) qI, quenching related to photoinhibition and with relaxation kinetics in the order of hours.

High energy state quenching qE is considered to be the main mechanism for photoprotection of plants *in vivo*. The mechanism of qE is still a matter of debate. One theory suggests that the processes, which determine the formation of qE are, associated with conformational changes in the pigment bed of LHC of PSII which cause the non-radiative dissipation (qE) of light energy (Fig. 1.3; Bilger and Björkman, 1994).
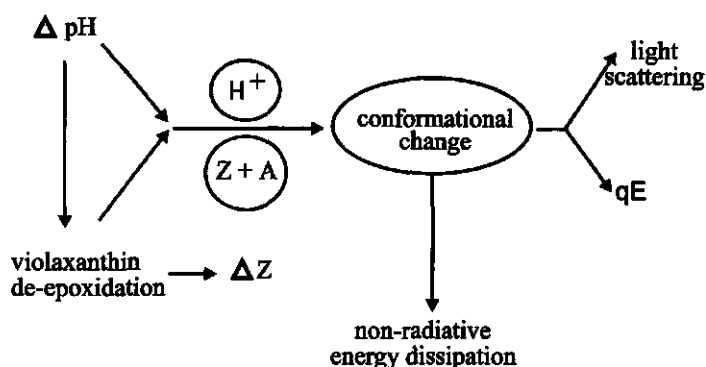
Fig. 1.3. Hypothetical scheme indicating possible functional relationships among proton concentration (ΔpH, H⁺), violaxanthin de-epoxidation, zeaxanthin (ΔZ), light scattering and high-energy state quenching (qE) (adapted from Bilger and Björkman, 1994).

## Xanthophyll cycle

In addition to the chlorophylls, different carotenoids are components of the pigment-protein complexes in the thylakoid membrane of the chloroplast. Xanthophylls make up 10 to 40% of the total carotenoids in leaves, depending on species and growth conditions (Rockholm and Yamamoto, 1996). Sapozhnikov *et al.* (1957) were the first to discover that the amount of violaxanthin in leaves was decreased by changing from dark to light in a reversible way.

Carotenoids are involved in the process of energy dissipation (Young, 1991). However, the mechanisms by which these carotenoids act in the dissipation of light energy is still a matter of discussion (Gilmore and Yamamoto, 1993; Ruban *et al.*, 1992; Demmig-Adams and Adams, 1996). Zeaxanthin is one of the carotenoids of the xanthophyll cycle, which has been found to be present in the LHCII (minor, peripheral complexes). Like qE, the concentration of zeaxanthin depends on the existence of a ΔpH across the thylakoid membrane (Fig. 1.3). The question is whether zeaxanthin itself is directly involved in the mechanism of photoprotection or whether zeaxanthin acts indirectly as a tuner (Ruban *et al.*, 1994), which in the presence of a transthylakoid proton gradient gives rise to conformational changes in the pigment bed (Fig. 1.4). In the first case this carotenoid may act by scavenging of triplet chlorophyll or singlet oxygen. The latter can be formed by the triplet state of chlorophyll and becomes excited after which it decays to its ground state under the emission of heat (Young, 1991). Initially, there was disagreement about a link between zeaxanthin and

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energy dissipation due to the lack of knowledge of a mechanism that allowed energy transfer from excited single chlorophyll to zeaxanthin. This mechanism was considered thermodynamically impossible (Siefermann-Harms, 1987). However, recent developments have revealed that a simple and direct downhill energy transfer is actually possible from excited singlet chlorophyll to zeaxanthin (Frank *et al.*, 1994). When zeaxanthin acts as a tuner, the second case, conformational changes might transform the antenna complexes of PSII in an efficient quencher of absorbed photons (Fig. 1.4). These conformational changes would facilitate non-radiative energy dissipation in the pigment bed, thereby lowering the fluorescence yield and simultaneously increasing the light-scattering properties of the thylakoid membrane (Bilger and Björkman, 1994).
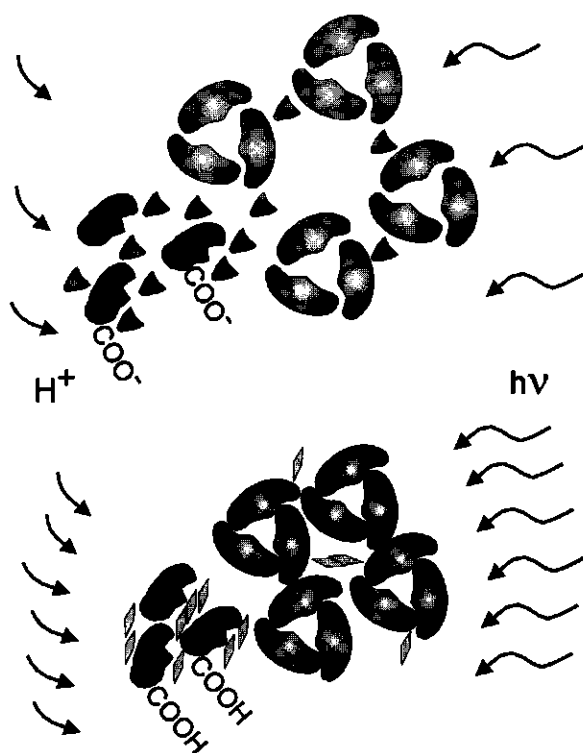


Fig. 1.4. Model to describe the proposed changes in LHCII associated with qE. Change from low irradiance (top) to high irradiance (bottom) results in increased proton concentration ($H^+$) in the thylakoid lumen. Protonation of the carboxyl residues ($COO^-$) on the minor LHCII ( ) and the de-epoxidation of violaxanthin ( ) to zeaxanthin ( ) results. Quenching, an increase in non-radiative dissipation of energy (Shown as a decrease in brightness of the LHCII), is associated with a change in organisation of LHCII, including LHCIIb ( ) and results from increased proximity of either chls or xanthophyll and chl (Horton *et al.*, 1994).

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Lumen acidification is caused by proton translocation during electron transport. It is required for the activation of the de-epoxidase enzyme (VDE) which regulates the de-epoxidation of violaxanthin to zeaxanthin. While this enzyme is still unidentified, the gene which codes for the zeaxanthin epoxidase (that turns zeaxanthin back to violaxanthin) has been identified. It is a gene that is involved in the abscisic acid biosynthesis (Marin *et al.*, 1996).

Apart from the role of zeaxanthin in the induction of non-radiative energy dissipation, other functions have also been ascribed to this carotenoid of the xanthophyll cycle. It is possible that epoxy carotenoids and/or zeaxanthin are structurally involved in the stacking of thylakoids (grana) and in the assembly of functional complexes of LHCII (Rock *et al.*, 1992). Furthermore, the presence of zeaxanthin appears to change the mechanical properties of lipid membranes at the peripheral region of the hydrophobic core (Gruszecki and Strzalka, 1991). As an accessory pigment, zeaxanthin plays a role in the light harvesting (Pfündel and Strasser, 1994). An additional effect of zeaxanthin is the stabilisation of the D1 protein by means of energy dissipation, minimising inactivation and consequently preventing degradation of D1 protein (Thiele *et al.*, 1996).

## Photoinhibition

When a plant receives excessive amounts of light, so that it cannot convert or dissipate it without harm, it undergoes a process called photoinhibition. The precise mechanism of this process is still intensively being studied. No uniform name for this event has been defined (Ball and Wild, 1993). Some research groups use the terminology photodamage or photodegradation (Aro *et al.*, 1993a), while others discuss the process of down regulation (Van Wijk *et al.*, 1993). Most researchers agree that this process can lead to extensive damage of the photosynthetic apparatus. Even under field conditions, the process of photoinhibition occurs as a common phenomenon and can possibly be intensified by anthropogenic environmental factors, *e.g.* air pollution (Krause, 1988). This process, in turn, can considerably reduce crop productivity (Ögren, 1988). Two recent reviews on photoinhibition are published by Prasil *et al.* (1992) and Aro *et al.* (1993b).

Photosystem II (PSII, Fig. 1.2) is regarded as the major target for the photoinhibitory damage (Van Rensen, 1993). Although photosystem I (PSI) has been looked upon as being resistant to high irradiance, photoinhibitory effects have also been reported for this part of the electron transport chain (Sonoike, 1995). However, PSII-dependent electron transport is much more sensitive to photoinhibition than PSI-dependent electron transport (Critchley, 1981; Cornic and Miginiac-Maslow, 1985; Havaux and Eyletters, 1991). The vulnerability of both photosystems for high irradiance has its consequences for the electron transport chain.

The involvement of the D1 protein turnover in the process of photoinhibition is still a matter of debate (Aro *et al.*, 1993a). The D1 protein is turning over continuously. The turnover rate of the D1 protein is already saturated at low irradiance (Critchley and Russell, 1994). Sundby (1993) found that the rate of turnover depends on the irradiance level during growth. D1 protein turnover is the balance between D1 degradation and synthesis; the co-ordination seems to be mediated by D1 protein phosphorylation (Rintamäki *et al.*, 1996). Research is directed towards the question whether the breakdown of D1 protein is occurring due to a direct effect of active oxygen species (Okada *et al.*, 1996) or due to a indirect effect by a cleavage via a D1 protein specific protease after the protein is attacked by active oxygen (Aro *et al.*, 1993b).

The irradiance level during growth has a major effect on the amount of pigments, especially chlorophyll *a* and *b*, and therefore on the absorption cross section of LHCII (Walters and Horton, 1995). Alterations of the thylakoid structure (antenna size of PSII) affect the sensitivity of the thylakoid membrane to photoinhibitory treatment (Krause, 1988). The irradiance level during growth of triazine-resistant (R) and susceptible (S) plants has a significant effect on the differences between the two biotypes in their sensitivity towards photoinhibition (Curwiel *et al.*, 1993).

In recent reports it was documented that *in vitro* R and S, using isolated broken chloroplasts, do not differ in their sensitivity to photoinhibition while *in vivo* R suffers more damage (lower Fv/Fm) than the S plants after a photoinhibitory treatment. The higher sensitivity of R to photoinhibitory treatment (PIT), *in vivo*, compared to S can be due to a difference in energy-dissipative mechanisms protective against photoinhibition (Curwiel and van Rensen, 1996).

The action of light in the process of photoinhibition is through photon exposure: the product of irradiance and duration of illumination. The number of photons absorbed by PSII, rather than the rate of absorption determines the extent of photoinactivation of PSII (Park *et al.*, 1996b).

# Outline of the study

The aim of this project was to study the relation between abiotic stress and triazine-resistance and their effects on photosynthesis. For this purpose two biotypes of *Chenopodium album* were used, a triazine-resistant and a -susceptible biotype. Preliminary research on this theme revealed that the two biotypes differ in their response to light irradiance during their growth period.

In the second chapter, experiments are described that were carried out *in vitro*, using isolated broken chloroplast suspensions. The study effect of photoinhibition on the photosynthetic capacity of triazine-resistant (R) and -susceptible (S) biotypes of *C. album* was studied. It appears that *in vitro* the two biotypes do not differ in their sensitivity to photoinhibitory treatment.

Chapter 3 deals with the same subject, described as above, but in a different context. Both biotypes were studied *in vivo* using intact leaves. Chlorophyll *a* fluorescence measurements were used as a non-invasive research tool to study the differences between the resistant and susceptible biotype. From the results a hypothesis was postulated; the difference in sensitivity between R and S to photoinhibition *in vivo* is due to a difference in energy-dissipative mechanisms which, in turn, can be protective against photoinhibition.

Elaborating on this theme, we investigated different energy dissipative pathways *in vivo*. The results are presented in chapter 4. Photoprotective pathways of the plant could be photorespiration, the operation of xanthophyll cycle or non-photochemical quenching.

To investigate how the plant reacts to photoinhibition, *in vivo*, both biotypes were subjected to photoinhibitory light after which the different components of non-photochemical quenching were determined (Chapter 5). qE is considered the main photoprotective mechanism to photoinhibition *in vivo*. In addition to these experiments, different inhibitors of photoprotective pathways were added to the leaves to examine the effect of these inhibitors on the sensitivity of both triazine-resistant and susceptible biotypes to photoinhibition (Chapter 6).

Finally, in chapter 7 the results from the different experiments are combined and discussed to gain a clear picture about the differences and similarities in photosynthesis and energy dissipative pathways of triazine-resistant and susceptible plants of *C. album*.

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**CHAPTER 2**


# INFLUENCE OF PHOTOINHIBITION ON ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION OF ISOLATED CHLOROPLASTS

Victor B. Curwiel and Jack J.S. van Rensen

**Abstract**

The effects of a photoinhibition treatment (PIT) on electron transport and photophosphorylation reactions were measured in chloroplasts isolated from triazine-resistant and susceptible *Chenopodium album* plants grown under high and low irradiance. Electron transport solely dependent on photosystem I (PSI) was much less affected by PIT than that dependent on both photosystem II (PSII) and PSI. There was a smaller difference in susceptibility to PIT between the photophosphorylation activity dependent on PSI and that dependent on both PSII and PSI. In all cases photophosphorylation activity decreased faster upon PIT than the rate of electron transport. Therefore, it was concluded that photoinhibition causes a gradual uncoupling between electron transport and phosphorylation. Also, the extent of the light-induced proton gradient across the thylakoid membrane decreased upon PIT. As a result, it was suggested that photoinhibiton causes a proton leakiness of the membrane. No significant differences were found in sensitivity to PIT of the various reactions measured in chloroplasts isolated from triazine-resistant and susceptible plants. In addition, no significant differences in sensitivity to PIT of the photophosphorylation reactions in chloroplasts of plants grown under low irradiance, compared with those grown under high irradiance. However, the electron transport reactions in chloroplasts from plants grown under low irradiance appeared to be somewhat less sensitive to PIT than those grown under high irradiance.

**Introduction**

Photoinhibition of photosynthesis occurs when plants are exposed to an irradiance level that is higher than they can convert or dissipate without harm via the available physiological and biochemical (-physical) routes. Former process is generally the case when a plant is exposed to a higher irradiance than experienced during growth. Photosystem II (PSII) is usually considered the major target for the photoinhibitory damage (for a review, see Prasil *et al.*, 1992). Although to a lesser extent, photoinhibition also affects the reaction center of photosystem I (PSI; *e.g.* Satoh, 1970; Inoue *et al.*, 1986; Yokoyama *et al.*, 1991) by destruction of the iron-sulphur centers by some species of active oxygen produced by illuminated chloroplasts. Because PI and herbicides are likely to share the core D1 protein of PSII as a major target, plant phenotypes with an altered herbicide sensitivity are of interest for study in the mechanisms of PI.

In the D1 protein of triazine-resistant plants, the serine at site 264 is replaced by glycine. This replacement results in a slower electron transport rate between $Q_A$ and $Q_B$ (Jansen et al., 1986; Jansen and Pfister, 1990). Therefore, it has recently been suggested that resistant plants are more susceptible to photoinhibition (Hart and Stemler, 1990; Van Rensen et al., 1990).

The irradiance during the growth period determines the amounts of chlorophyll a and b and the size of the light-harvesting complex of photosystems I and II (Anderson, 1986). Former changes can affect the susceptibility of the thylakoid membrane to a photoinhibition treatment (Krause, 1988).

In this study, the kinetics of the decrease in PSII and/or PSI-dependent electron transport activities upon a photoinhibitory treatment have been compared. Because few studies exist on the effect of photoinhibition on energetic processes in thylakoids, investigations on photophosphorylation reactions have been included. These reactions were measured in thylakoids isolated from triazine-resistant and susceptible *Chenopodium album* plants, grown under high and low irradiance.

## Materials and methods

### *Plant material*

The area of collection of seeds, the methods of germination, the transplantation of seedlings, the patterns and conditions of growth of *Chenopodium album* L. plants were described previously (Jansen et al., 1986). Three weeks after germination, the plants were placed in a mixture of black soil and fine quartz sand and transferred to another growth chamber where they were grown under two different irradiances. The temperature in this growth chamber was controlled at 22°C, the light period was 16 h per day and the relative humidity was about 60%.

### *Irradiance during growth*

The plants were grown under two irradiance levels: high (HI), *i.e.* 120 W·m$^{-2}$ (PAR) and low (LI), *i.e.* 30 W·m$^{-2}$ (PAR). To obtain these irradiances, TL-tubes (Philips) TLMF 140 W/ 33 RS were used.

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*Isolation of chloroplasts*

Three weeks after the beginning of the light treatment the uppermost leaves were harvested. Broken chloroplasts were isolated as described by Snel and Van Rensen (1983). The chloroplasts (washed grana suspension) were suspended in a final solution with a medium containing 0.4 M sorbitol, 20 mM Tricine, pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$, and 2 g $l^{-1}$ bovine serum albumin. The chlorophyll content was determined according to Bruinsma (1963) and the chloroplasts stored at -80°C. All experiments were conducted with thawed chloroplasts.

*Photoinhibition treatment*

Broken chloroplasts isolated from triazine-resistant and susceptible plants grown under HI and LI, were given different periods of pre-illumination with photoinhibitory irradiation (470 $W \cdot m^{-2}$ PAR). Chloroplast suspensions containing 25 µg $Chl \cdot ml^{-1}$ were used for both the measurements of electron transport and photophosphorylation. The assay media for both types of photoinhibition treatments (PIT) were as described below with some modifications. With the electron transport, the electron donors and/or acceptors were omitted during the period of pre-illumination. In the case of photophosphorylation, ADP and $K_2HPO_4$ were omitted during the pre-illumination period. At the end of the PIT, the appropriate reagents were added to ensure the continuation of electron and photophosphorylation reactions rates. By comparing the activity after a period of photoinhibitory exposure with the same time period of darkness, the effect of PIT was measured and expressed as percentage of the dark control activity.

*Measurement of electron transport*

Electron transport was measured as oxygen exchange as described by Van Rensen *et al.* (1977). The assay medium of the Hill reaction ($H_2O \rightarrow FeCy$) contained: 0.3 M sorbitol, 50 mM Tricine, pH 7.6, 5 mM $MgCl_2$, 5 mM $NH_4Cl$ and 1 mM FeCy. The rate of this reaction was usually about 180 µmol $O_2$ $mg \cdot Chl^{-1} \cdot h^{-1}$.

Two PSI-dependent electron transport systems were studied. The assay medium for the Mehler reaction with methyl viologen (MV) as the electron acceptor and ascorbate/DCPIP as electron donor (DCPIP $\rightarrow$ MV) contained: 0.3 M sorbitol, 50 mM Tricine, pH 7.6, 5 mM $MgCl_2$, 1 µM DCMU, 2 mM dithioerythritol, 2 mM ascorbate, 40 µM DCPIP and 20 µM MV.

The rates of these reactions were typically 160 $\mu$mol $O_2$ mg·Chl$^{-1}$·h$^{-1}$. The Mehler reaction was also studied by using DAD as electron donor (DAD $\rightarrow$ MV) instead of ascorbate/DCPIP. The assay medium in this experiment contained the same components as in the first Mehler reaction except that 500 $\mu$M DAD was used instead of ascorbate/DCPIP. Typical reaction rates were about 600 $\mu$mol $O_2$ mg·Chl$^{-1}$·h$^{-1}$.

## *Measurement of photophosphorylation*

Photophosphorylation was measured as light-induced pH changes observed at pH 8 (Mills, 1986). The phosphorylation reactions were carried out as described by Van Rensen and Hobé (1979) in the same water-jacketed chamber which was used for the oxygen exchange measurements. Proton consumption was measured with an Ingold glass electrode and monitored with a Philips pH meter (Model PW9420) equipped with a ABB recorder (Model SE 120). The reaction temperature was maintained at 25°C.

Non-cyclic photophosphorylation dependent on both PSII and PSI was measured with MV as electron acceptor ($H_2O \rightarrow$ MV). The assay medium contained 0.3 M sorbitol, 100 mM KCl, 5 mM MgCl$_2$, 100 $\mu$M MV, 1 mM K$_2$HPO$_4$ and 1 mM ADP with initial pH $\pm$ 8.0. The averaged rates at light saturation were about 230 $\mu$mol ATP mg Chl$^{-1}$·h$^{-1}$.

Two PSI-dependent phosphorylation reactions were studied. The first one was measured using PMS as cofactor. The assay medium contained the same components as in the non-cyclic photophosphorylation reaction except that 20 $\mu$M PMS was used instead of MV. Typical rates were 580 $\mu$mol ATP mg·Chl$^{-1}$·h$^{-1}$. In the second PSI-dependent phosphorylation reaction, DAD was added as an electron donor and MV as an electron acceptor (DAD $\rightarrow$ MV). The assay medium contained the same components as in the non-cyclic photophosphorylation reaction, except that 500 $\mu$M DAD was added as an electron acceptor and 1 $\mu$M DCMU to inhibit the electron transport from PSII. The averaged reaction rates at light saturation were about 430 $\mu$mol ATP mg Chl$^{-1}$·h$^{-1}$.

## *Measurement of light-induced proton gradient*

In a separate experiment, the light-induced proton gradient across the thylakoid membrane was measured with the same set-up as that for the measurement of

photophosphorylation. The light-induced steady-state proton gradient ($\Delta pH$) was measured at an external pH of 6.0 in a medium containing 100 mM KCl, 5 mM $MgCl_2$ and 20 $\mu M$ PMS.

**Results**

In order to show the effects of a photoinhibition treatment on electron transport reactions more clearly, the data from the four types of plant material (RHI, SHI, RLI and SLI) were averaged (Fig. 2.1). Electron transport involving both PSII and PSI ($H_2O \rightarrow FeCy$) decreased more rapidly than electron transport dependent on PSI alone (DCPIP $\rightarrow$ MV). After a PIT of about 15 min no activity of PSII-dependent electron transport was left, while about 30% activity remained of the PSI-dependent rate, even after 25 min PIT.
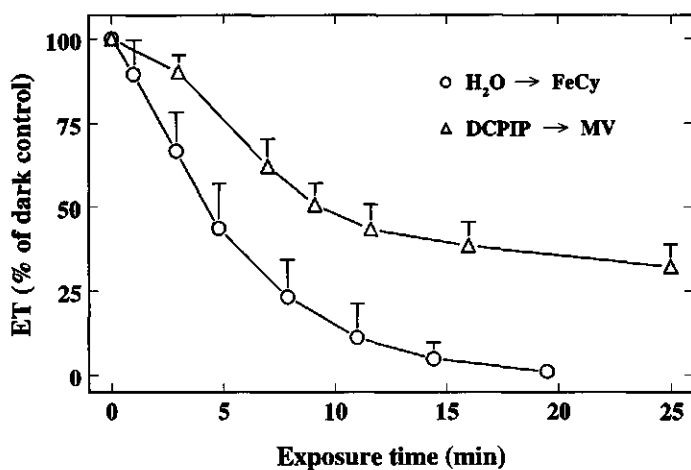


Fig. 2.1. The effect of photoinhibition treatment on electron transport (ET) dependent on PSII and PSI ($H_2O \rightarrow FeCy$) or on PSI alone (DCPIP $\rightarrow$ MV) of isolated chloroplasts. Vertical bars indicate $\pm$ SD (n = 8).

Table 2.1. Half time values (min) of the decrease of the electron transport and photophosphorylation reactions in chloroplasts isolated from *Chenopodium album* upon photoinhibition treatment and the remaining activity (% of dark control) of the different photochemical reactions after photoinhibitory (PI) treatment. RHI, SHI = chloroplasts from high irradiance grown triazine-resistant or susceptible plants; RLI, SLI = chloroplasts from low irradiance grown triazine-resistant or susceptible plants, respectively. Values represent means ± SD (n = 2). Please note that the total duration of PIT is not the same for the different reactions.

| Half time value | RHI | SHI | RLI | SLI |
|---|---|---|---|---|
| *Electron transport* | | | | |
| $H_2O \rightarrow FeCy$ | 3.6 ± 0.1 | 3.7 ± 0.8 | 4.0 ± 0.6 | 5.7 ± 1.4 |
| $DCPIP \rightarrow MV$ | 8.7 ± 0.0 | 7.7 ± 1.0 | 11.6 ± 2.6 | 11.2 ± 1.2 |
| $DAD \rightarrow MV$ | 4.2 ± 0.3 | 4.0 ± 0.0 | 5.0 ± 0.0 | 5.1 ± 0.4 |
| *Photophosphorylation* | | | | |
| $H_2O \rightarrow MV$ | 4.5 ± 1.2 | 3.7 ± 0.9 | 3.8 ± 0.1 | 4.8 ± 3.5 |
| $DAD \rightarrow MV$ | 4.3 ± 0.4 | 3.9 ± 0.2 | 3.8 ± 0.6 | 3.8 ± 0.3 |
| PMS | 2.0 ± 0.4 | 1.6 ± 0.3 | 2.5 ± 0.3 | 2.3 ± 0.6 |
| % activity | RHI | SHI | RLI | SLI |
| *Electron transport* | | | | |
| $H_2O \rightarrow FeCy$ | 1.3 ± 1.2 | 1.1 ± 0.0 | 0.7 ± 0.6 | 1.8 ± 0.0 |
| $DCPIP \rightarrow MV$ | 27.8 ± 7.8 | 29.6 ± 8.6 | 36.6 ± 3.9 | 35.2 ± 6.2 |
| $DAD \rightarrow MV$ | 24.6 ± 2.1 | 19.0 ± 2.2 | 26.1 ± 4.0 | 22.9 ± 1.4 |
| *Photophosphorylation* | | | | |
| $H_2O \rightarrow MV$ | 0.9 ± 1.3 | 13.8 ± 7.3 | 1.5 ± 2.1 | 10.5 ± 2.2 |
| $DAD \rightarrow MV$ | 19.4 ± 2.8 | 17.5 ± 0.2 | 15.7 ± 7.0 | 17.6 ±.0.1 |
| PMS | 2.5 ± 2.1 | 2.0 ± 1.0 | 1.1 ± 1.6 | 0.9 ± 0.1 |

The PSI-dependent activity with DAD as an electron donor also decreased comparatively slower and was not completely inhibited due to PIT as compared with that dependent on PSII (Table 2.1).

The average effects of PIT on photophosphorylation reactions of the four plant materials (RHI, SHI, RLI and SLI) were estimated (Fig. 2.2). The photophosphorylation activity dependent on both PSII and PSI ($H_2O \rightarrow MV$) decreased fast upon photoinhibition and after

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about 10 min the rate was completely inhibited. The PSI-dependent cyclic photophosphorylation with PMS as a cofactor (PMS) decreased even faster than the PSII + PSI-dependent phosphorylation (Fig. 2.2). In the case of the PSII and PSI dependent photophosphorylation ($H_2O \rightarrow MV$) S has under both conditions (HI; LI) a much higher final percentage of activity than R (Table 2.1), suggesting a lower sensitivity of S to PIT compared to R. The PSI dependent phosphorylation (DAD $\rightarrow$ MV) shows in all treatments similar kinetics in percentage of remaining activity due to PIT in comparison to the electron transport rates (Table 2.1).
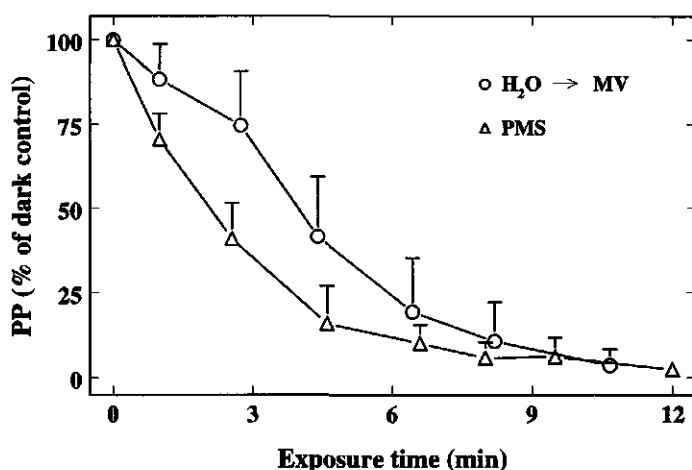


Fig. 2.2. The effect of photoinhibition on photophosphorylation (PP) dependent on PSII and PSI ($H_2O \rightarrow MV$) or on PSI alone (PMS) of isolated chloroplasts. Vertical bars indicate ± SD (n = 8).

Comparing the decrease of electron transport activity due to PIT with that of phosphorylation it appeared that under most conditions phosphorylation decreased faster than electron transport (Table 2.1). Therefore, we investigated the effect of PIT on the coupling between electron transport and phosphorylation (Fig. 2.3). Because there was little difference between triazine-resistant (R) and triazine-susceptible (S) plants, R and S were averaged. Studying the system DCPIP $\rightarrow$ MV, the electron transport rate was measured in the absence (coupled) and in the presence (uncoupled) of CCCP.
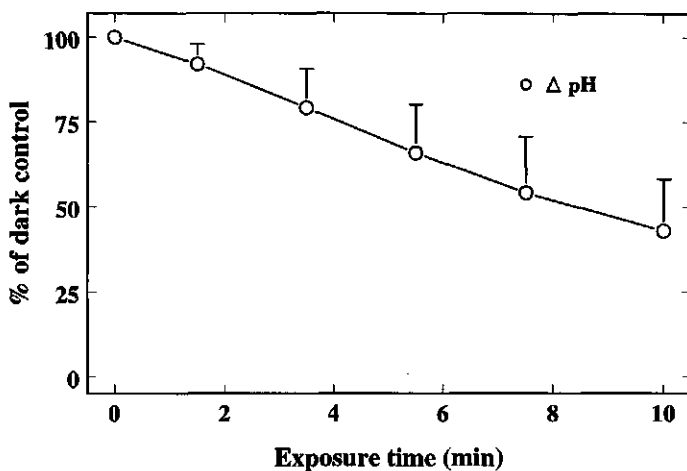
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Fig. 2.3. The effect of photoinhibition treatment on the coupling of electron transport with phosphorylation of chloroplast isolated from plants, grown under high (HI) and low (LI) irradiance. The coupling ratio was determined as the rate of electron transport +CCCP over that -CCCP. Vertical bars indicate ± SD (n = 4).
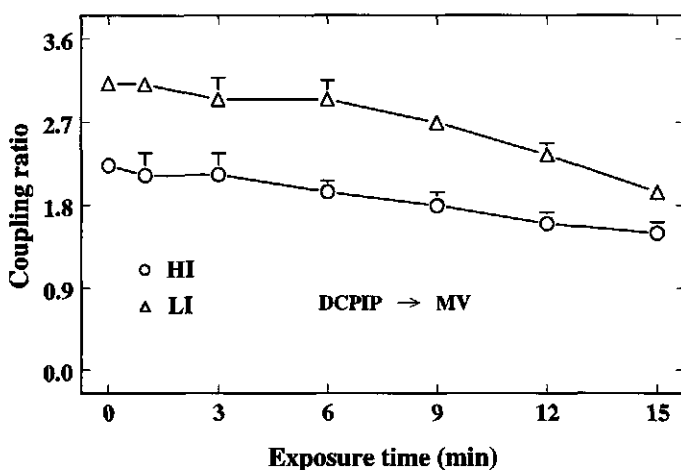


Fig. 2.4. The effect of photoinhibition treatment on the light induced proton gradient (ΔpH) of isolated thylakoid membranes. Vertical bars indicate ± SD (n = 4).

The coupling ratio appeared to decrease gradually with time during photoinhibitory treatment. In a separate experiment we found a PIT-induced loss of the proton gradient across the thylakoid membrane (Fig. 2.4). The results indicate no significant differences in half time of the decrease in both electron transport and phosphorylation reactions due to photoinhibition in chloroplasts from R and S plants (Table 2.1).

The PSI dependent electron transport reactions in thylakoids isolated from plants grown under low irradiance were less susceptible to photoinhibitory light of equal fluorescence rate than those from plants grown under high irradiance (Table 2.1). We found no significant differences in sensitivity to PIT of the phosphorylation reactions in chloroplasts from plants grown under low or high irradiance (Table 2.1), except for the PSII and PSI-dependent photophosphorylation ($H_2O \rightarrow MV$) which shows under both irradiance conditions less inhibition in the S plants after photoinhibitory treatment than in the R plants.

**Discussion**

Photosystem II-dependent electron transport is more susceptible to photoinhibition than PSI-dependent electron transport (Fig. 2.1, Table 2.1). This is in accordance with earlier reports (Critchley, 1981; Cornic and Miginiac-Maslow, 1985; Havaux and Eyletters, 1991; Wu *et al.*, 1991). A residual activity of PSI-dependent electron transport remains even after long exposure times (*e.g.* Fig. 2.1, DCPIP $\rightarrow$ MV), indicating that part of the PSI electron transport is susceptible to PIT; the other part of PSI is tolerant to PIT.

Photoinhibition has a strong effect on the photophosphorylation (PP) reactions of the thylakoid membrane (Fig. 2.2, Table 2.1). Cyclic PP with PMS appeared to be very susceptible to photoinhibition. The half time of the decrease of the rate of the PMS-catalysed PP due to PIT were about 2-fold lower than those of the other PP reactions (Table 2.1). It was reported earlier by Garber (1977) that PMS-catalysed PP is one of the thylakoid reactions which is most sensitive to photoinhibition. Very recently, Tjus and Andersson (1992) also found a rapid deactivation of the PMS-mediated cyclic· photophosphorylation after photoinhibitory illumination.

Comparing the kinetics of the decay of the various reactions due to photoinhibition, it is noticeable that the electron transport reactions are generally less susceptible to PIT than the

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phosphorylation reactions. Studying the effect of PIT on the coupling ratio of ET with PP of the reaction asc/DCPIP → MV, we found a decrease during PIT exposure (Fig. 2.3). This means that the chloroplasts become gradually uncoupled during PIT. Comparable results were recently reported by Tjus and Andersson (1992). Two earlier studies reported no effect of PIT on the coupling factor. Critchley (1981) studied electron and phosphorylation reactions in chloroplasts isolated from cucumber leaves before and after PIT. Photoinhibition resulted in inhibition of PSII electron transport and non-cyclic PP of about 50%, cyclic PP was less inhibited and light-induced proton uptake was unaffected. It was concluded that PIT has no effect on the coupling factor and that light stress does not result in alteration of the membrane properties. Barényi and Krause (1985) reported that PIT of broken chloroplasts inhibited coupled non-cyclic ET at the same degree as the concurrent PP. The ATP/2e$^-$ ratio remained constant and the authors concluded that there is no uncoupling of PP as a result of light stress.

The gradual uncoupling observed in my present study could be explained by an effect of PIT either on the coupling enzyme (ATPase) or on the integrity of the thylakoid membrane, resulting in leakage of protons and an attenuation of proton motive force. It was found by Garber (1977) that light-dependent proton uptake by thylakoids was more sensitive to PIT than the ATPase activity, while Jung and Kim (1990) observed that the chloroplast $CF_0$-$CF_1$ ATPase is about 10-fold less sensitive to PIT than electron transport. The results of our measurements on the effects of PIT on the light-induced proton gradient across the thylakoid membrane (Fig. 2.4) indicate that PIT impairs the integrity of the thylakoid membrane, resulting in leakage of protons and uncoupling. However, when the kinetics of the decrease of PMS-mediated proton gradient and the PMS-mediated cyclic photophosphorylation are compared, it appears that PP is earlier affected than the proton gradient. This means that very probably also the ATP-synthase is affected by PIT.

Based on earlier reports (Hart and Stemler, 1990; Van Rensen et al., 1990) we had expected differences in the sensitivity to PIT between R and S plants. In these studies intact leaves have been used. In the present investigations (Table 2.1) we used isolated chloroplasts and found no significant differences in sensitivity to PIT with only one exception (PP of $H_2O$ → MV).

We have observed no significant differences in PIT susceptibility of the PP reactions in chloroplasts of plants grown under low irradiance, compared with those grown under high irradiance (Table 2.1). However, the ET reactions in chloroplasts from plants grown under

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low irradiance appeared to be somewhat less sensitive to PIT than those from plants grown under high irradiance (Table 2.1). This is in contradiction to what is generally observed: shade-grown plants are more susceptible to photoinhibition than high light grown plants (Bhogal and Barber, 1987; Tyystjärvi *et al.*, 1991).

It is generally assumed that sensitivity to PIT is affected by (1) the rate of photosynthetic energy conversion and by energy dissipation by protective mechanisms (Wu *et al.*, 1991); (2) the rate of recovery; (3) the size of the light-harvesting antenna (Krause, 1988). Growth at low irradiance induces larger light-harvesting antenna of PSII than growth in high irradiance (Anderson, 1986) and thus it may be expected that shade-grown plants are more sensitive to PIT. Tyystjärvi *et al.* (1991) found indeed that low-light leaves were more susceptible to PIT *in vivo* than high-light leaves. However, thylakoids isolated from these two plant materials appeared to be equally sensitive to PIT. The differential effect of PIT *in vivo* and in vitro was suggested to be due to the fact that *in vitro* energy conversion by photosynthesis, energy dissipation by protective mechanisms and recovery processes do not function. This is probably the reason why I find no or small differences in sensitivity to PIT in chloroplasts isolated from low-light plants compared with those isolated from high-light plants, and in addition no significant differences in sensitivity to PIT in chloroplasts isolated from triazine-resistant and susceptible plants (Curwiel and Van Rensen, 1993).

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**Chapter 3**

# COMPARISON OF PHOTOSYNTHETIC ACTIVITIES IN TRIAZINE-RESISTANT AND SUSCEPTIBLE BIOTYPES OF *CHENOPODIUM ALBUM*

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**Abstract**

Triazine-resistant and susceptible *Chenopodium album* plants were grown at low and at high irradiance. At the lower irradiance the dry matter production of the resistant and the susceptible plants was almost similar. At the higher irradiance the resistant biotype had a significantly lower production. Fluorescence studies showed that the photochemical yield and the photosystem II electron transport rate were lower in the resistant biotype. It could be demonstrated in intact leaves that the lower productivity of the resistant biotype is caused by a higher sensitivity to photoinhibition. However, when studying effects of photoinhibition on electron flow and photophosphorylation in isolated thylakoids of the two biotypes, no significant differences between resistant and susceptible plant materials were observed. It is suggested that the differences between resistant and susceptible biotypes connected with processes protective against photoinhibition in intact leaves, are lost during the isolation of thylakoids.

**Introduction**

Triazine resistance of several plant species is caused by an alteration in the D1 protein of photosystem II (PSII). At site 264, serine is replaced by glycine (Hirschberg *et al.,* 1984; Trebst, 1991; Van Rensen, 1992). This alteration causes resistance to atrazine and other members of the triazine family (Naber and Van Rensen, 1991; Van Rensen and De Vos, 1992). There appears a small resistance to diuron-type herbicides, while sensitivity to phenol-type herbicides is increased (Pfister and Arntzen, 1979; Jansen *et al.*, 1986).

The alteration of the D1 protein does not only result in triazine resistance, but also in a decrease of the binding affinity for plastoquinone in the $Q_B$-binding niche in the D1 protein (Bowes *et al.,* 1980). This causes a 3-fold decrease in the rate of electron flow from $Q_A^-$ to $Q_B$ (Jansen and Pfister, 1990). It was suggested that the changed kinetics of the $Q_A/Q_B$ reaction do not simply decrease primary photosynthetic efficiency via a direct effect on photosynthetic electron flow, but that the mutation in the D1 protein affects also other functional aspects of the PSII complex important for regulation of photosynthesis and biomass production, *e.g.* the turnover of the D1 protein (Jansen and Pfister, 1990).

The triazine resistance trait has been transferred to several crop plants, *e.g.* rapeseed (*Brassica napus* L.), Chinese cabbage (*Brassica campestris* L.) and foxtail millet (*Setaria italica* L.). Unfortunately, a significant reduction in yield accompanies the resistance trait in most species studied. Field studies with resistant rapeseed demonstrated decreased growth and crop yield. The above-mentioned slower rate of electron flow between $Q_A$ and $Q_B$ has been suggested as the cause of the reduction in photon yield, maximum photosynthesis and ecological fitness (*e.g.* Holt *et al.*, 1981; Ireland *et al.*, 1988).

An influence of the impaired electron transport between $Q_A$ and $Q_B$ on the overall electron transport rate was questioned by Jansen *et al.* (1986). The lower rate of electron flow between $Q_A$ and $Q_B$ in the resistant biotype is still about 20 times faster than the oxidation of reduced plastoquinone. The latter reaction having a half-time of about 20 ms remains the rate limiting step. These authors demonstrated that the electron transport between water and plastoquinone has a lower rate and a lower quantum yield in isolated chloroplasts of resistant plants. However, no significant differences were found for the rate and quantum yield of whole chain electron transport.

It has recently been suggested that the differences (Chapter 1) between resistant (R) and susceptible (S) plants occur only when grown at high light irradiance and are much less when grown at low irradiance (Hart and Stemler, 1990a; Van Rensen *et al.*, 1990; Hart and Stemler, 1990b). Hart and Stemler (1990a) compared R and S *Brassica napus* plants, grown under low photon flux density. They found that the slow electron flow from $Q_A$ to $Q_B$ was still present in the R biotype, but photon yield and light-saturated oxygen evolution were similar in the two *B. napus* biotypes. These authors proposed (Hart and Stemler, 1990b) that the differential reduction in photon yield and photosynthesis often observed in R biotypes when plants are grown at high photon flux density is the result of an increased sensitivity to photoinhibition.

We measured growth of R and S biotypes of *Chenopodium album* plants grown at high (HL) and low (LL) light irradiances. Intact leaves of R plants showed lower photochemical quenching, lower photochemical yield and lower PSII electron flow rate compared to leaves of S plants. HL leaves of R biotypes appeared to be more sensitive to photoinhibition than HL leaves of S biotypes, the differences between the LL plants were much less. However, thylakoids isolated from R and S plants of both HL and LL show no significant differences in their sensitivity for photoinhibition. Possible reasons for the difference in sensitivity for photoinhibition in leaves compared with that in isolated thylakoids of R plants are discussed.

**Materials and Methods**

The growth from seed to seedling of triazine-resistant (R) and susceptible (S) biotypes of *Chenopodium album* L. was previously described (Jansen *et al.*, 1986). Three weeks after germination the seedlings were placed under two different irradiances: a high light irradiance (HL) of 120 $W \cdot m^{-2}$ (PAR) and a low light irradiance (LL) of 30 $W \cdot m^{-2}$ (PAR) for 16h/day during a period of three weeks. The dry matter of the above ground parts of R and S biotypes was determined for plants grown at both irradiances.

Different parameters of chlorophyll *a* fluorescence were measured (Van Kooten and Snel, 1990). Whole leaves of both biotypes of *C. album* grown at the two irradiances were used. Measurements of modulated chlorophyll fluorescence emission from the upper surface of the leaf were made using a pulse amplitude modulation fluorometer (PAM-101) (Schreiber *et al.*, 1986). These measurements were carried out at about 25 °C. Furthermore, a photoinhibition treatment (PIT) was carried out, consisting of a period of preillumination with photoinhibition light (800 $W \cdot m^{-2}$, PAR) at 5 °C for 15 min after which the $F_v/F_m$ values were determined. These values were compared with the dark control (period of 15 min in the dark at 5 °C).

After growing R and S *Chenopodium album* plants for three weeks, chloroplasts were isolated ( Snel and Van Rensen, 1983) and stored at -80 °C. Electron transport was measured as oxygen exchange (Van Rensen *et al.*, 1977). The Hill and the Mehler reaction were used to measure the PSII and/or the PSI dependent electron transport. Starting at pH 9, cyclic and non-cyclic photophosphorylation was measured as light-induced pH changes in the presence of an ATP-forming system. The photoinhibitory treatment of these two types of measurements comprised different periods of preillumination with strong light (470 $W \cdot m^{-2}$, PAR). At the end of the preillumination period the appropriate reagents were added to measure the rates of electron flow and photophosphorylation reactions.

**Results and Discussion**

When triazine-resistant (R) and susceptible (S) *Chenopodium album* plants were grown at low light irradiance (LL) the dry matter production of R and S plants was almost similar. At the higher light irradiance (HL) the R biotype showed a significantly lower productivity (Table 3.1).

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Table 3.1. Dry weight production of resistant (R) and susceptible (S) *Chenopodium album* plants grown at high light (HL) and at low light (LL) irradiances.

| RHL | SHL | RLL | SLL |
|---|---|---|---|
| $1.14 \pm 0.10$ | $1.65 \pm 0.21$ | $0.09 \pm 0.02$ | $0.11 \pm 0.03$ |

Dry matter in grams per plant. HL is 120 $W \cdot m^{-2}$; LL is 30 $W \cdot m^{-2}$ (PAR).

Fluorescence studies with intact leaves did not show significant differences (qP, qN, J and $\Phi_p$) under HL and LL conditions between R and S (Figs. 3.1-3.4). Nevertheless, the values of the R leaves were always lower than those of the S leaves with a slight tendency that the differences were somewhat larger at the HL conditions.
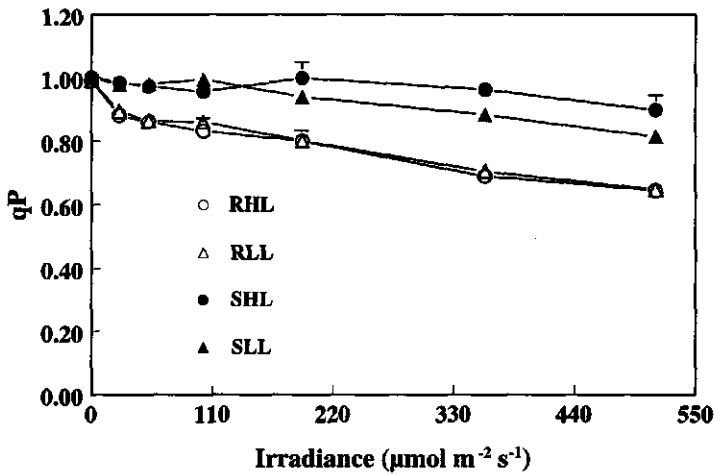


Fig. 3.1. Photochemical quenching (qP) at different irradiances of intact leaves of triazine-resistant (R) and susceptible and (S) plants of *Chenopodium album*, grown under high light (HL) and low light irradiances.

Although, as previously reported (Chapter 2), the overall electron flow rate of R and S was similar, the rate of PSII dependent electron flow in chloroplasts isolated from R plants has

been reported to be lower than that in S chloroplasts (Jansen *et al.*, 1986). This could be confirmed by the fluorescence measurements, illustrated in Figs. 3.1-3.4. Photochemical quenching (qP) was lower for the R leaves at all irradiances (Fig. 3.1). This indicates that for equal irradiances the fraction of open PSII reaction centers of R is always lower than of S. A lower fraction of open reaction centers may induce a higher probability of absorbed photons to be dissipated as heat, *i.e.* increase of non-photochemical quenching (qN). Actually, a higher qN was only found for R leaves grown at HL, measured at low actinic irradiances. However, at higher irradiances and at all irradiances for the LL leaves, qN appeared to be lower for R than for S leaves (Fig. 3.2).
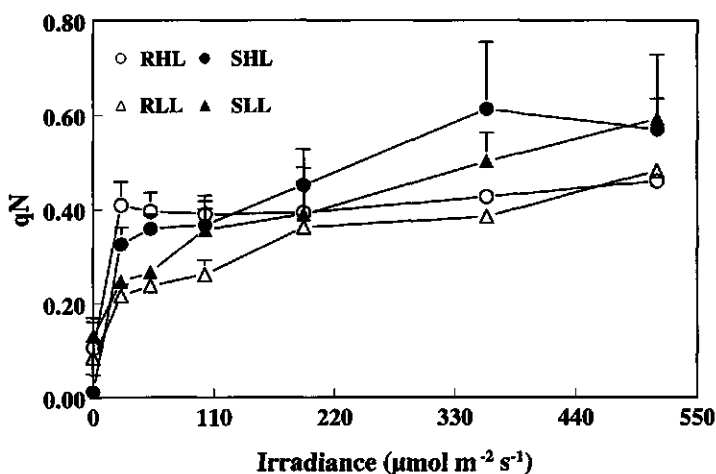


Fig. 3.2. Non-photochemical quenching (qN) at different irradiances of intact leaves of R and S plants of *C. album*, grown under high light (HL) and low light (LL) irradiances.

Apparently, the lower rate of PSII electron flow in R plants, leads to a lesser amount of proton production at the water splitting site, especially at higher irradiances. This may cause a smaller proton gradient and may contribute to a lower qN. The quantum yield of PSII electron flow ($\phi_P$) was lower for R leaves at both HL and LL conditions (Fig. 3.3). From the fluorescence data the rate of PSII dependent electron flow (J) could be calculated: the R leaves exhibited lower rates than the S leaves at all irradiances (Fig. 3.4). Similar results from fluorescence
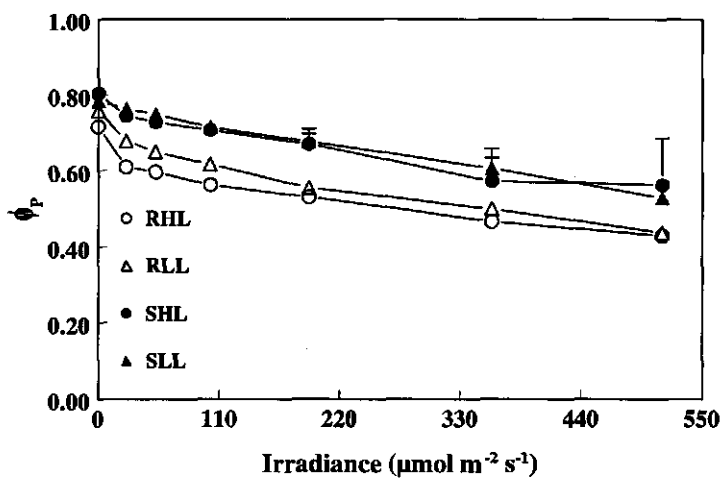
Fig. 3.3. Photochemical quantum yield of PSII electron flow ($\phi_P$) at different irradiances of intact leaves of R and S plants of *C. album*, grown under high light (HL) and low light (LL) irradiances.

studies using R and S *Brassica napus* plants were recently reported by Sundby *et al.* during the 9th Int. Congress of Photosynthesis at Nagoya (Sundby *et al.*, 1992).
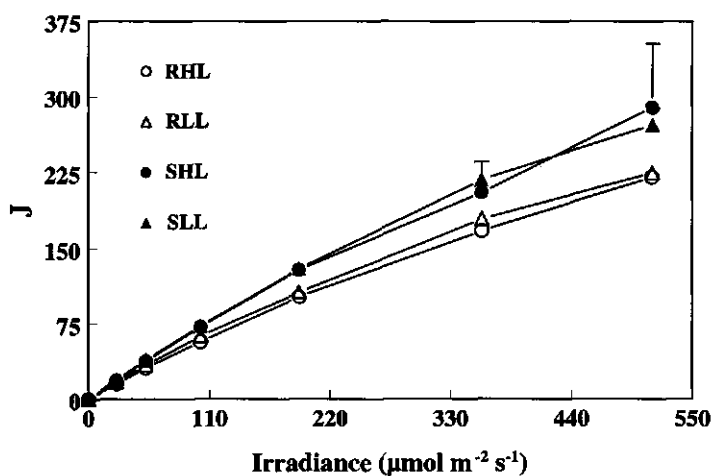


Fig. 3.4. Photosystem II electron transport rate (J) at different irradiances of intact leaves of R and S plants of *C. album*, grown under high light (HL) and low light (LL) irradiances.

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While the results of the fluorescence measurements confirm earlier reports (*e.g.* Jansen *et al.*, 1986) that PSII electron flow in R proceeds at a lower rate than in S plants, there was little difference between the HL and LL conditions with respect to PSII performance. The lower dry matter production of R plants at HL (Table 3.1) must, therefore, be due to other processes. Because the lower electron flow rate between $Q_A$ and $Q_B$ induces a more reduced state of $Q_A$ in R plants, it is very probable that R plants are more sensitive to photoinhibition, especially when grown at high irradiance. The effects of a photoinhibitory treatment of intact leaves is illustrated in Table 3.2. Although plants adapted to high light suffer somewhat less from photoinhibition, it appears that the R biotype is indeed more sensitive to photoinhibition than the S plants. The difference is less when grown at low irradiance.

Table 3.2. Photoinhibition measured as $F_v/F_m$ values of leaves of R and S plants, grown under HL and LL irradiances.

|      | RHL | SHL | RLL | SLL |
|------|-----|-----|-----|-----|
| Dark | $0.80 \pm 0.01$ | $0.85 \pm 0.01$ | $0.81 \pm 0.01$ | $0.84 \pm 0.01$ |
| PIT  | $0.58 \pm 0.02$ | $0.69 \pm 0.04$ | $0.51 \pm 0.03$ | $0.58 \pm 0.04$ |

$F_v/F_m$ was measured after the leaves were kept at 5 °C during 15 min, while one half of the leaf was darkened (dark) and the other half illuminated at 800 $W \cdot m^{-2}$ (PIT).

When chloroplasts were isolated from R and S plants and subjected to a photoinhibitory treatment (PIT), no significant differences in chloroplast activities were observed between R and S biotypes. The treatment of PIT was examined in more detail by measuring effects on three types of PSII and/or PSI dependent electron transport reactions (Fig. 3.5). In all three cases there was no significant difference between R and S biotypes. In Fig. 3.6 results are presented of effects of PIT on PSII and/or PSI dependent photophosphorylation reactions. Remarkable is the very fast decrease of the PSI dependent cyclic photophosphorylation with PMS during photoinhibition. But also in this case there appeared no significant differences between R and S.
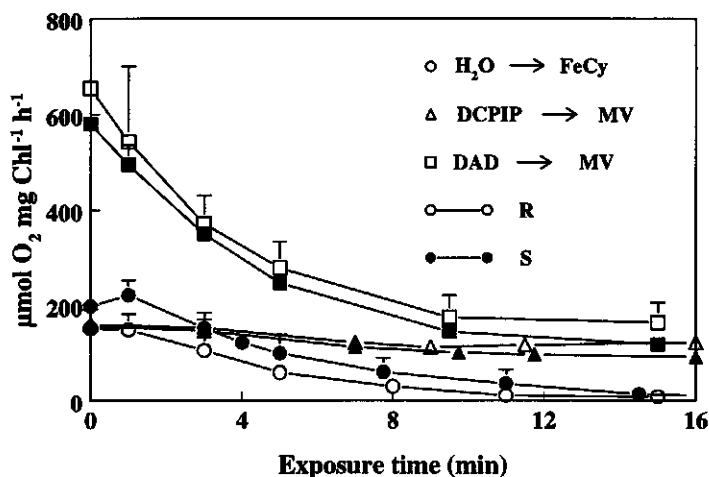
Fig. 3.5. Effect of photoinhibition treatment on the PSII and/or PSI dependent electron transport of chloroplasts isolated from R (open symbols) and S (closed symbols) plants. The assay and reaction media were as described in chapter 2. Photoinhibition light was 470 W·m$^{-2}$ (PAR). Please note that the values in the figure above are not corrected for changes in the dark controls.
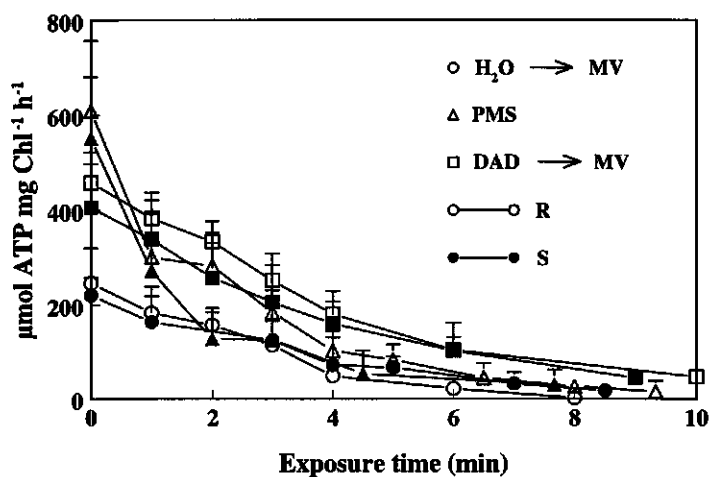


Fig. 3.6. Effect of photoinhibition treatment on the PSII and/or PSI dependent photophosphorylation reactions of chloroplasts isolated from R (open symbols) and S (closed symbols) plants. For further details see Fig. 3.5.

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*In vitro* R en S do not differ much in their sensitivity to photoinhibition (Chapter 2), while *in vivo* R is more prone to photoinhibitory damage than S at equal fluence rate (Table 3.2). This may be caused by the fact that *in vitro* energy conversion by photosynthesis, energy dissipation by protective mechanisms and recovery processes do not function or function less efficiently. The higher sensitivity of R biotypes *in vivo* must be due to one or more of these processes, which are all protective against photoinhibition. It is important to mention that Sundby *et al.* (1992) reported that the D1 protein turnover rate was different in R biotypes of *Brassica napus* compared with that in the S plants. In general, it appears that differences between R and S biotypes observed in intact leaves may not be present in chloroplasts isolated from them.

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# CHAPTER 4

## CHLOROPHYLL FLUORESCENCE QUENCHING, ZEAXANTHIN FORMATION AND LIGHT SCATTERING IN INTACT LEAVES OF TRIAZINE-RESISTANT AND SUSCEPTIBLE *CHENOPODIUM ALBUM* PLANTS

Victor B. Curwiel and Jack J.S. van Rensen

**Abstract**

The triazine-resistant biotype of *Chenopodium album* has an impaired activity of photosystem II. *In vivo*, this leads to a higher sensitivity to photoinhibition, especially when the plants have been grown at a high irradiance. The activities of several protecting energy dissipation pathways were compared in triazine-resistant and susceptible biotypes which were grown at low and high irradiances.

It was found that the resistant plants have more light-induced zeaxanthin formation and a larger change in light scattering than does the susceptible biotype. The differences were more pronounced when the plants were grown at a high irradiance. Stern-Volmer plots indicated that high light-grown plants show a linear relationship between non-photochemical quenching (qN) and zeaxanthin content, and also between qN and change in light scattering. In low light-grown plants, the zeaxanthin content and the change in light scattering are not linearly related to qN and there is a strong increase in the value of qN already at low levels of zeaxanthin content and change in light scattering. The latter effect is smaller in the resistant biotype. Photorespiration is more important as an energy dissipation pathway in resistant plants compared with susceptible plants.

It is concluded that the increased sensitivity to photoinhibition of resistant plants is not caused by a lower activity of the photoprotective pathways, including qN, zeaxanthin formation and photorespiration. Instead, it is suggested that the shade-type characteristics of the chloroplasts of the resistant plants are responsible for the greater sensitivity to photoinhibition.

**Introduction**

In triazine-resistant (R) plants the serine at site 264 of the D1 protein is replaced by glycine. As a consequence of this alteration these plants are resistant to triazine-type herbicides (Naber and Van Rensen, 1991) and in addition, have a lower rate of electron transport between $Q_A$ and $Q_B$ of PSII (Jansen and Pfister, 1990). Resistant plants have a lower rate of dry matter production than do triazine-susceptible (S) plants when grown at high light irradiance (Hart and Stemler, 1990; Rensen *et al.*, 1990). It was recently demonstrated that in intact leaves the lower productivity of the R biotype is caused by a greater sensitivity to photoinhibition (Chapter 2; Chapter 3; Sundby *et al.*, 1993).

Adverse effects on the photosynthetic apparatus as photoinhibition and photodamage occur when the capacity of photoprotection of the plant is exceeded. Possible mechanisms of photoprotection are reflected by fluorescence quenching (Ruban *et al.*, 1993), the activity of the xanthophyll cycle (Demmig-Addams and Addams III, 1992) and photorespiration (Wu *et al.*, 1991). The resistant plants are reported to have more and larger grana, more LHC associated with PSII (LHCII) and a lower amount of chloroplast coupling factor (Vaughn, 1986; Burke *et al.*, 1982; Vaughn and Duke, 1984). While qP may be lower in the R plants because of the mutation in the D1 protein, there may also be differences between the two biotypes in zeaxanthin formation and qN.

The non-photochemical fluorescence quenching (qN) results from competing de-excitation processes of the excited state of Chl associated with PSII. Several processes in the thylakoid membrane contribute to qN *in vivo*: qE, qT and qI. There are strong correlations between the extent of quenching and the activity of the xanthophyll cycle: the de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin (Demmig-Addams and Adams III, 1992). The role of these carotenoids in the dissipation of light energy is still a matter of debate (Ruban *et al.*, 1993; Gilmore and Yamamoto, 1993). One possibility is that the xanthophyll cycle acts indirectly as a tuner (Ruban *et al.*, 1994) which, in the presence of a trans-thylakoid proton gradient, induces conformational changes in the pigment bed of LHCII. These conformational changes, which transform the antenna complexes of PSII in an efficient quencher of absorbed photons, are paralleled by increases in light scattering (Bilger and Björkman, 1994).

Searching for the cause of the increased sensitivity of triazine-resistant plants to photoinhibition, we measured the capacities of energy dissipation pathways in R and S plants: qP, qN, zeaxanthin formation and photorespiration.


## Materials and methods

### *Plant material*

The origin and the growth conditions of the *Chenopodium album L.* plants were described previously (Chapter 2-3, see also Jansen *et al.*, 1986). Three weeks after germination the plants were placed in a mixture of black soil and fine quartz sand (4:1) and transferred to other growth chambers were all plants were grown under the same conditions: the temperature

was controlled at 20°C, the light period was 16 h per day and the relative humidity was about 70%. The plants were grown under two different irradiances: high (HI), *i.e.* 480 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR) and low (LI), *i.e.* 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR). As the light source TL-tubes (Philips) were used: TLD 58 W/ 84.

### *Measurement of fluorescence*

Chlorophyll *a* fluorescence was measured *in vivo*; whole leaves of both biotypes of *C. album* were used while still attached to the plant. First, the plants were placed in an almost dark room and the leaves were dark-adapted in the fluorometer for at least 5 min. Measurements of modulated chlorophyll fluorescence emission from the upper surface of the leaf were made using a pulse amplitude modulated fluorometer (PAM-101, H. Walz, Effeltrich, Germany). During the measurements, the leaves were humidified with moisturised air, containing 2% $O_2$ (325 ppm $CO_2$) for the control measurements and 20% $O_2$ (317 ppm $CO_2$) for the determination of photorespiration effects. All these measurements were carried out at about 20 °C. The actinic irradiance varied from about 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to about 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and the measurements were started with the highest irradiance. Calculations of the Chl fluorescence quenching parameters qP and qN were made according to Van Kooten and Snel (1990), $\phi_P$ of PSII electron transport was calculated as (Fm' - F)/Fm', while the activity of PSII electron transport (ET) was calculated from fluorescence parameters using the method of Snel *et al.* (1991). In some experiments the Stern-Volmer coefficient of quenching ($SV_N$) was used with $SV_N = Fm/Fm' - 1$.

### *Measurement of absorbance*

The changes in carotenoid contents of the leaves of R and S *Chenopodium album* L. plants were determined by measuring the absorbance change at 505 nm, indicative of the de-epoxidation of the xanthophyll pigment violaxanthin to zeaxanthin (Yamamoto and Kamite, 1972; Yamamoto *et al.*, 1972). The reference wavelength was 535 nm. The experiments were thermostated at a temperature of about 25 °C. The absorbance changes were measured with an AMINCO DW-2a UV/visible spectrophotometer (American Instrument Company, MD, USA) in the dual beam mode (halfband-width, 3.0 nm). The actinic light beam was filtered through a red filter (RG 630)

and the irradiance varied from about 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ to about 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The photomultiplier was shielded by a blue filter (BG 39; 5 mm thickness). After harvesting, the intact leaves were put between two layers of aluminium foil with wet tissue paper for dark adaptation for 2 - 5 h to reduce background levels of zeaxanthin and antheraxanthin. After this dark adaptation, each leaf was cut to the appropriate size to fit in a glass cuvette (diameter, 1 cm) and positioned at an angle of 45° to the measuring and the actinic light beam. When a stable baseline was achieved the actinic light source was turned on and the absorbance changes at 505 nm were measured. After the absorbance change reached its maximum (after 5 to 10 min) the light was turned off and 3-5 min after the rapid relaxation the total amount $\Delta Z$ of light-induced zeaxanthin at 505 nm was determined (Fig. 4.1).
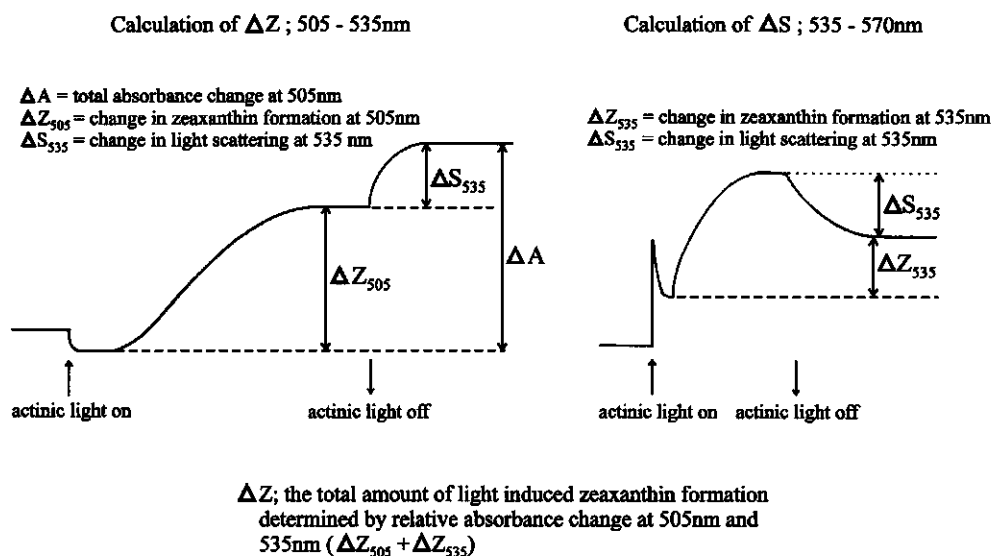


Calculation of $\Delta Z$ ; 505 - 535nm          Calculation of $\Delta S$ ; 535 - 570nm

$\Delta A$ = total absorbance change at 505nm
$\Delta Z_{505}$ = change in zeaxanthin formation at 505nm
$\Delta S_{535}$ = change in light scattering at 535 nm

$\Delta Z_{535}$ = change in zeaxanthin formation at 535nm
$\Delta S_{535}$ = change in light scattering at 535nm

$\Delta Z$; the total amount of light induced zeaxanthin formation
determined by relative absorbance change at 505nm and
535nm ($\Delta Z_{505} + \Delta Z_{535}$)

Fig. 4.1. Calculation of the different absorbance components ($\Delta Z$ and $\Delta S_{535}$) by measuring the absorbance changes at 505-535nm and at 535-570nm.

According to recent findings (Bilger and Björkman, 1994), $\Delta Z$ can have a substantial contribution to the total absorbance at 535 nm. Our measurements showed also that the contribution of zeaxanthin at 535 nm was present in both biotypes, and varied between 20% and 30% of $\Delta Z$ (Fig. 4.1). Therefore the zeaxanthin amount was also measured at 535 nm sampling wavelength and 570 nm as the reference wavelength. The change in absorbance measured at these wavelengths consists of two components: a large change in light scattering and a small

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$\Delta Z$. The two components can be separated by turning off the light after the absorbance has reached its maximum; 3-5 min later the change in light scattering is relaxed and $\Delta Z$ remains. $\Delta Z$ was calculated by adding the small component determined at 535 nm to the larger component measured at 505 nm (Fig. 4.1).

The change $\Delta S_{535}$ in light scattering of the leaves was determined from the rapid relaxation of the light scattering at 535 nm (reference wavelength, 570 nm). It was measured 3-5 min after turning off the light when the absorbance change had reached its maximum. The change in light scattering is the difference between the maximum and the dark level (Fig. 4.1).

Although all plants were grown under the same conditions except for the irradiance level, the absorbance changes may be influenced by differences in morphological, anatomical and ultrastructural features of the leaves. Therefore, many leaves were measured and the result averaged.
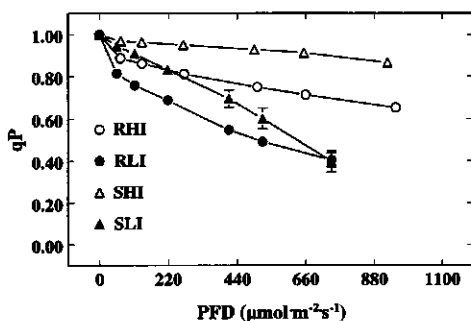
## Results



Fig. 4.2. Photochemical quenching (qP) as a function of PFD of leaves of triazine-resistant (R) and susceptible (S) *Chenopodium album* plants, grown under high (HI) and extreme low (LI) irradiances measured in an atmosphere of 20% $O_2$. Each value represents the mean of three different HI experiments or five different LI experiments. Bars indicate $\pm$ SD; SD bars not shown are within the symbols.
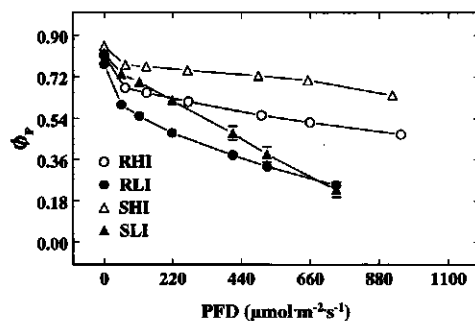
Fig. 4.3. Photochemical quantum yield of PSII electron transport ($\phi_P$) as a function of PFD of leaves of triazine-resistant (R) and susceptible (S) *C. album* plants, grown under high (HI) and extreme low (LI) irradiances measured in an atmosphere of 20% $O_2$. For further details see legend Fig. 4.2.

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The level of photochemistry and the activity of dissipating processes appear to respond to the irradiance level at which the plant is grown. Compared with the LI-grown plants, the HI-grown plants have a higher qP, $\phi_P$ and rate of electron flow, ET (Figs. 4.2-4.4 and Table 4.1).

Table 4.1. Fluorescence parameters (qN, qP, $\phi_P$) and electron flow rate (ET) measured in intact leaves of triazine-resistant (R) and susceptible (S) *C. album* plants where the plants were grown under either HI or LI and the measurements were done in the presence of a 2% $O_2$ or 20% $O_2$ atmosphere (each value is measured at the highest actinic irradiance (for LI plants, 740 $\mu mol \cdot m^{-2} \cdot s^{-1}$; for HI, 940 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and represents the mean of three different HI experiments or five different LI experiments; with the ± SD in parentheses.

| | RHI 2% $O_2$ | RHI 20% $O_2$ | SHI 2% $O_2$ | SHI 20% $O_2$ |
|---|---|---|---|---|
| qN | 0.67 (0.03) | 0.43 (0.02 | 0.59 (0.07) | 0.46 (0.04) |
| qP | 0.63 (0.02) | 0.65 (0.00) | 0.79 (0.04) | 0.87 (0.01) |
| $\phi_P$ | 0.38 (0.00) | 0.47 (0.01) | 0.54 (0.06) | 0.64 (0.01) |
| ET | 352.8 (4.7) | 446.5 (5.7) | 509.7 (57.0) | 589.6 (13.3) |
| | **RLI** 2% $O_2$ | **RLI** 20% $O_2$ | **SLI** 2% $O_2$ | **SLI** 20% $O_2$ |
| qN | 0.55 (0.03) | 0.59 (0.03) | 0.73 (0.02) | 0.71 (0.01) |
| qP | 0.28 (0.02) | 0.40 (0.03) | 0.37 (0.03) | 0.40 (0.05) |
| $\phi_P$ | 0.17 (0.02) | 0.25 (0.02) | 0.21 (0.02) | 0.23 (0.03) |
| ET | 127.4 (11.5) | 185.1 (18.1) | 152.4 (17.5) | 171.2 (24.5) |

The HI-grown plants have a lower value of non-photochemical quenching qN is lower for PFD above 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Fig. 4.5). The LI-grown plants have a lower photochemical activity, as indicated by a lower qP, $\phi_P$ and ET (Figs. 4.2-4.4 and Table 4.1).
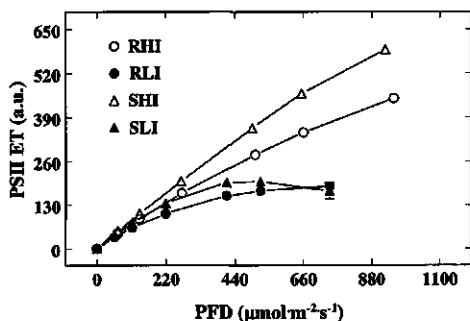
Fig. 4.4. Photosystem II electron transport rate (ET) as a function of the PFD of leaves of triazine-resistant (R) and susceptible (S) *C. album* plants, grown under high (HI) and extreme low (LI) irradiances measured in an atmosphere of 20% $O_2$. For further details see legend Fig. 4.2.
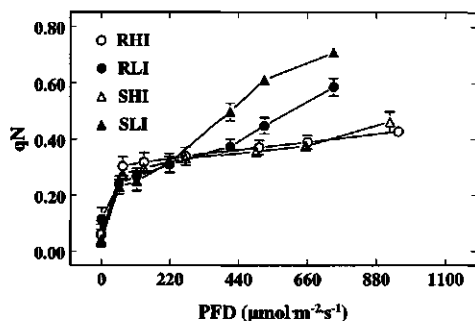
Fig. 4.5. Non-photochemical quenching (qN) as a function of the PFD of leaves of triazine-resistant (R) and susceptible (S) *C. album* plants, grown under high (HI) and extreme low (LI) irradiances measured in an atmosphere of 20% $O_2$. For further details see legend Fig. 4.2.

The triazine-resistant *C. album* plants also have a lower qP, $\phi_P$, and ET than the susceptible plants, especially when grown under HI and measured at 20% $O_2$ as found in chapter 3 (Figs. 4.2-4.4). The differences between qN for R and S are only significant for the LI-grown plants (Fig. 4.5): S has a higher qN than R at high actinic irradiance, which enables S to dissipate light energy more non-radiatively. Comparing the 2% $O_2$ measurements with those at 20% $O_2$ (Table 4.1) it appears that the effect on qN is substantial in HI-grown plants which suggests an effect on photorespiration. Only when the photochemistry is working near maximum (high qP) does the presence of an extra sink for electrons (photorespiration) compete with qN for energy which results in a lower qN (Krall and Edwards, 1992). This effect is much larger for the R than for the S biotype. The presence of an extra electron sink plants has a stronger increasing effect on ET in R plants compared with S plants when oxygen levels are increased from 2 to 20% (Tabel 4.1).

In addition to a higher capacity for photochemistry, a HI during growth also leads to higher amounts of convertible zeaxanthin, for both R and S (Fig. 4.6). It is known that shade plants have a lower amount of zeaxanthin (Johnson *et al.*, 1993), which can also be seen from our results. From Fig. 4.6 it appears further that the LI-grown plants have already reached their

maximal light-induced zeaxanthin formation at about 600 µmol·m$^{-2}$·s$^{-1}$ (PAR), while the HI-grown plants, especially the S biotype, have not yet reached the maximum at 1000 µmol·m$^{-2}$·s$^{-1}$. No significant differences between R and S were found for the LI-grown plants. However, when grown at HI, large differences between R and S were observed, especially at the light levels up to which the plants were grown (480 µmol·m$^{-2}$·s$^{-1}$); R has a higher zeaxanthin formation than S at actinic irradiances below 700 µmol·m$^{-2}$·s$^{-1}$ (Fig. 4.6).
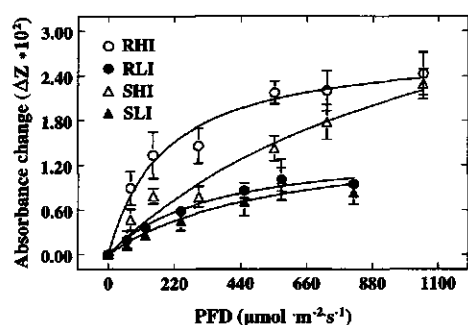


Fig. 4.6. Relative absorbance changes measured at 505 nm and 535 nm, indicative for the total de-epoxidation of violaxanthin to zeaxanthin ($\Delta Z$) as a function of the PFD in intact leaves of triazine-resistant (R) and susceptible (S) C. album plants, grown under high (HI) and extreme low (LI) irradiances. Each value represents the mean of six different HI measurements or five different LI measurements. Bars indicate ± SD; SD bars not showing are within the symbols.
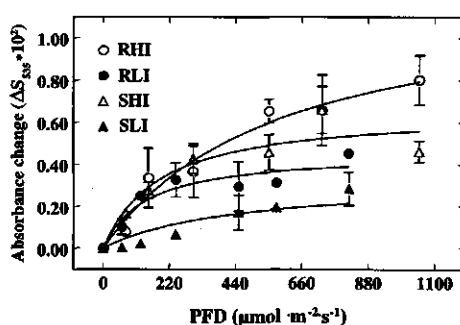
Fig. 4.7. Relative absorbance changes measured at 535 nm, indicative for the change in light scattering ($\Delta S_{535}$) as a function of the PFD in intact leaves of triazine-resistant (R) and susceptible (S) C. album plants, grown under high (HI) and extreme low (LI) irradiances. For further details see Fig. 4.6.

Changes in light scattering are illustrated in Fig. 4.7. The HI-grown plants show a higher level of change in light scattering than do the LI-grown plants. A higher level of scattering is indicative for higher membrane energization (Bilger et al., 1988). The differences between R and S are not significant for the HI-grown plants for PFD below 400 µmol·m$^{-2}$·s$^{-1}$. On the contrary, the differences between $\Delta S_{535}$ for R and S with the LI-grown plants are significant, R having more light scattering than S (Fig. 4.7). When compared with the measurements of qN for the LI-grown

plants (Fig. 4.2), the R biotype has a high $\Delta S$, but low qN at high actinic irradiance, while the S biotype tends to show a low $\Delta S$ and a high qN. When $\Delta Z$ is plotted against $\Delta S_{535}$ (Fig. 4.8), it appears that the relations for both R and S are linear when grown under HI as well as under LI.
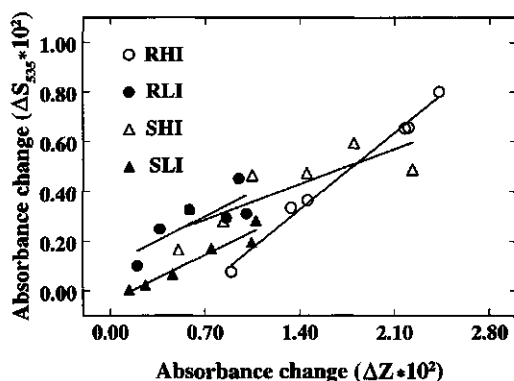


Fig. 4.8. Relationship between total zeaxanthin formation and change in light scattering in intact leaves of triazine-resistant (R) and susceptible (S) *C. album* plants, grown under high (HI) and extreme low (LI) irradiances. For further details see legend Fig. 4.6.



Fig. 4.9. Relationship between total zeaxanthin formation and SV$_N$ in intact leaves of triazine-resistant (R) and susceptible (S) *C. album* plants, grown under high (HI) and extreme low (LI) irradiances. For futher details see legend Fig. 4.6.

Fig. 4.10. Relationship between change in light scattering and SV$_N$ in intact leaves of triazine-resistant (R) and susceptible (S) *C. album* plants, grown under high (HI) and extreme low (LI) irradiances. For further details see legend Fig. 4.6.
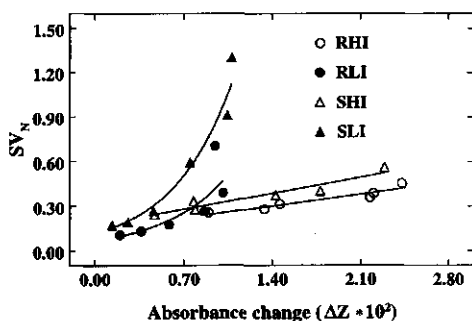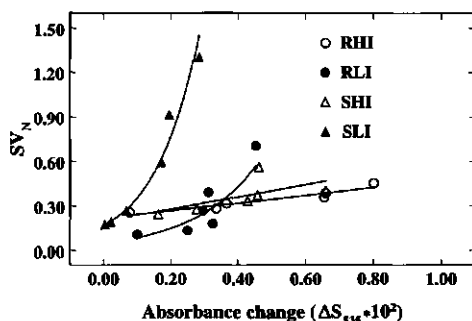
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Using the Stern-Volmer equation, the amount of zeaxanthin formation and the change in light scattering were plotted as a function of qN (Figs. 4.9 and 4.10). In the HI-grown plants there is a close to linear relationship between $\Delta Z$ and qN. The relation is altered in the plants grown at LI, at a certain point, $\Delta Z$ reaches a maximum while qN is still increasing (Fig. 4.9). Relating the changes in light scattering to qN resulted for the HI-grown plants in the same almost linear relationship for both R and S (Fig. 4.10). We found that growing the plants at LI alters this relationship in the same way as for $\Delta Z$.

## Discussion

The sensitivity of plants to photoinhibition *in vivo* is influenced by the light irradiance level during the growth period. Shade-grown plants develop more damage by photoinhibition than do plants grown at HI. Intact triazine-resistant (R) plants are more sensitive to photoinhibition than are susceptible (S) plants, especially when the plants are grown at high light levels (Chapter 3; Sundby *et al.*, 1993). The difference in sensitivity to photoinhibition may be caused by differences in (the efficiency of) the various photoprotective mechanisms. These differences may be related to the lower electron flow rate at the $Q_B$-site in the R biotype and, in addition, to the shade-type characteristics of the R chloroplasts (Vaughn, 1986; Burke *et al.*, 1982; Vaughn and Duke, 1984). We have measured the capacity of several light-energy-dissipating pathways in R and S plants, grown at LI and at HI irradiance levels: fluorescence quenching, light-induced zeaxanthin formation and also photorespiration.

Figs. 4.2-4.4 indicate that LI-grown plants always have a lower qP, $\phi_P$ and electron flow rate than HI-grown plants. This can be explained by the phenomenon that shade plants have fewer PSII centers and a larger antenna. This results in a higher level of reduction in $Q_A$ and a lower qP. With only one exception (LI-grown plants at the highest irradiance), qP, $\phi_P$ and the PSII electron flow rate is lower for R plants than for S plants. This is due to the mutation of the D1 protein in R; the R plants have a reduced PSII electron transport rate between $Q_A$ and $Q_B$.

From the fluorescence measurements of qN (Fig. 4.5) it appears that above a certain irradiance level the values of qN are lower for the HI-grown plants than for the LI-grown plants. This indicates that HI-grown plants can dissipate more light energy through photochemistry than

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can the LI-grown plants, leading to a lower qN (Johnson *et al.*, 1994). Of the LI-grown plants, R has a lower qN than S above a certain irradiance level. The latter result may be explained by including photorespiration as an energy-dissipative mechanism.

The effect of photorespiration on qN is larger in the HI-grown plants than in the LI-grown plants (Table 4.1). This effect of elevated $O_2$-levels is indicated by the higher qP and ET values of the HI-grown plants. Apparently, at high photochemical activity and ET, an extra sink, competes with qN for energy, resulting in a lower qN (Krall and Edwards, 1992). The decrease of qN due to photorespiration is larger for the R than for the S biotype. This indicates that R plants adapted to high light irradiance dissipate relatively more energy through photorespiration, thereby lowering energy dissipation *via* qN. Of the LI-grown plants the effect of photorespiration on photochemistry (qP, $\phi_P$ and ET) in S is less pronounced than in R (Table 4.1). It may be concluded that photorespiration has a lower activity in S than in R. This could imply a lower demand for ATP in the S plants causing a reduced relaxation of ΔpH, leading to a higher level of qN at high actinic irradiance.

In accordance with what was reported earlier (*e.g.* Johnson *et al.*, 1993) LI-grown plants have a lower zeaxanthin content than do HI-grown plants (Fig. 4.6). While there is little difference in zeaxanthin content of R and S biotypes when grown at LI, at HI conditions the R plants have more zeaxanthin than do the S plants. The change in light scattering of HI-grown plants is higher than that of the LI-grown plants, especially at the higher actinic irradiances (Fig. 4.7). The LI-grown R plants always show more scattering than do the S plants. Of the plants grown at HI, R has more scattering than S above a certain actinic irradiance level.

It has been reported that chloroplasts of the R plants contain relatively low amounts of chloroplast coupling factor (Vaughn, 1986). It was also reported that photosynthetic control in thylakoids of the R biotype is about half that in the S plants (Rashid and Van Rensen, 1987). Consequently, R thylakoids appear to utilise the ΔpH less efficiently for photophosphorylation than do S thylakoids. This can led to higher levels of zeaxanthin and changes in light scattering in R. Except for zeaxanthin of the LI-grown plants, this is indeed observed in Figs. 4.6 and 4.7. When plotting the extent of zeaxanthin against the change in light scattering in all cases almost linear relationships are observed (Fig. 4.8). This indicates that both zeaxanthin formation and change in light scattering react in the same way to a common cause, which is very probably ΔpH.

Almost linear relations were observed between both zeaxanthin content and qN, and between change in light scattering and qN in the R and the S biotype of the HI-grown plants (Figs. 4.9-4.10). This indicates that, under high irradiance, zeaxanthin formation and change in scattering are coupled in non-photochemical energy dissipation. Such relationships were also reported by Bilger and Björkman (1991), Ruban and Horton (1992) and Ruban et al. (1993). Extrapolation of the correlations to the origin shows that, for most cases, part of qN is correlated neither with the zeaxanthin content, nor with the changes in scattering. The difference form zero is more pronounced in HI plants. Possibly, the enlarged zeaxanthin content at HI (Fig. 4.6) leads to more free xanthophyll carotenoids, which are not bound to proteins, so that they cannot contribute to qN (Jahns and Krause, 1994). If part of the larger amount of zeaxanthin in R grown at HI is not directly involved in non-radiative dissipation, but acts as accessory pigments for light capture, this leads to overreduction of the electron transport chain and inactivation of the photosynthetic apparatus, especially at HI. The presence of qN at very low levels of zeaxanthin content and scattering change may also be explained by the zeaxanthin already present in the leaves of the HI-grown plants before the measurement of qN started, inducing qN already at very low light levels.

Of the plants grown at LI, all curves in Figs. 4.9 and 4.10 are non-linear and bend upwards, the curves of the S biotype bending at lower levels of zeaxanthin and scattering. The strong increase in qN in these LI-grown plants may be caused by their larger grana. Under excessive irradiation grana stacking allows the accumulation of photoinhibited PSII centers (Anderson and Aro, 1994). These dissipative PSII centers (Critchley and Russell, 1994) lead to qI being a larger part of qN. While the curves of R and S are not extremely different for the relation between zeaxanthin and qN (Fig. 4.9), there are large differences for the relation between scattering and qN (Fig. 4.10). The observation that for both biotypes the curve bends upwards at low levels of light scattering may be explained by the phenomenon that qN consists of several quenching components of which some are not related to the process of light scattering (e.g. qT and qI). In the case of S it is possible that these processes form a larger part of qN than for R, resulting in curve bending already at lower light irradiances.

The results of our studies on energy-dissipating pathways in triazine-resistant and susceptible plants grown at LI and HI indicate that shade plants have lower photochemical activity and more non-photochemical quenching than do high-light-adapted plants. Because the zeaxanthin content and the capacity of light scattering of shade plants are lower, the higher qN

in LI-grown plants is very probably caused by a larger qI, *i.e.* quenching due to photoinhibition. Analysis of qN into its separate components (qE, qT and qI) awaits further experimentation.

The greater sensitivity of triazine-resistant plants to photoinhibition compared with susceptible plants does not appear to be caused by lower activities of the photoprotecting pathways, including qN, zeaxanthin formation and photorespiration. Looking for another explanation, we wish to pay attention to the findings that, although the macrostructure of the leaves of R plants is similar to that of the S plants, the structure of the R chloroplasts is more alike that of a shade-adapted plant (Vaughn, 1986). Chloroplasts of R plants have more and larger grana, more LHC associated with PSII and a lower ratio of Chl *a* to Chl *b* (Vaughn, 1986; Burke *et al.*, 1982; Vaughn and Duke, 1984). While *C. album* is an extreme sun-type plant with a very low capacity for non-photochemical quenching (Johnson *et al.*, 1994), the R biotype apparently has developed chloroplasts with more shade-type characteristics. These shade-type chloroplasts thus apparently are more sensitive to photoinhibition.

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# CHAPTER 5


# FLUORESCENCE QUENCHING ANALYSIS IN TRIAZINE-RESISTANT AND SUSCEPTIBLE *CHENOPODIUM ALBUM* LEAVES IN RELATION TO PHOTOINHIBITION

**Abstract**

The triazine-resistant biotype of *Chenopodium album* has an impaired activity of photosystem II. *In vivo*, the resistant plants have shade-type chloroplasts and a higher sensitivity to photoinhibition, especially when the plants have been grown at high irradiance. The role of photochemical and non-photochemical quenching in the sensitivity of triazine-resistant and susceptible *Chenopodium album* to photoinhibition has been studied.

It was found that resistant plants (R) have a lower photochemical quenching, qP, compared to the susceptible plants (S). Similarly, the non-photochemical quenching, qN, was lower in the R leaves. The components making up qN were analysed: "high energy state" quenching, qE, quenching due to state transitions, qT, and quenching due to photoinhibition, qI. In the R leaves, qI was found to be lower than in the S leaves while there was almost no difference between R and S with regard to the qT. However, the qE was much lower in the R biotype.

The lower qP is explained by a larger absorbance cross section of photosystem II (PSII) in the shade-type chloroplasts of the R plants. This process enhances the odds of excitation of PSII through a higher excitation pressure by which more photons can reach the reaction center. In combination with a reduced rate of electron flow at the reducing side of PSII, former process cause a higher level of closed reaction centers and a reduced qP resulting in less photoprotection in R plants. The observed lower qE in the R biotype is suggested to be due to a lower PSII electron flow rate and a lower photosynthetic control in R compared with that in S, leading to a smaller proton gradient over the thylakoid membrane. The lower qE results in less non-radiative energy dissipation in the antenna pigment bed and a higher sensitivity to photoinhibition of the R plants.

**Introduction**

In triazine-resistant plants, the D1 protein of the photosystem II complex is altered at site 264 where the amino acid serine is replaced by glycine (Jansen *et al.*, 1986). The resistant mutant (R) differs in several aspects from the susceptible (S) plants: R plants have a lower electron transport rate between $Q_A$ and $Q_B$ and have shade-type chloroplasts. Another difference is that resistant biotypes are more sensitive to photoinhibition than the wild type. For a review, see Van Rensen and De Vos (1992).

If exposed to excess light energy, plants can protect themselves against photoinhibitory damage. One of the photoprotection mechanisms in chloroplasts involves non-radiative energy dissipation which is measurable as non-photochemical quenching, qN. The principle of this phenomenon has been studied since the light-induced reversible decrease in chlorophyll fluorescence has been discovered (Duysens and Sweers, 1963; Murata and Sugahara, 1969). qN results from the non-photochemical de-excitation of the singlet excited state of chlorophyll associated with PSII. This parameter can be separated into three components which usually have different relaxation kinetics. The first component is "high-energy state" quenching of fluorescence, qE, which is the one with the fastest relaxation kinetics. qE is dependent on the energization of the thylakoid membrane or, more specifically, the thylakoid $\Delta$pH. A second component, qT, has intermediate kinetics and it is related with state 1-state 2 transitions. The third component is the quenching due to photoinhibition, qI, it relaxes very slowly and normally persists after the dissipation of $\Delta$pH. The major component of qN is qE and it is caused by thermal dissipation of excess light energy in the pigment bed. qE is considered to be an important mechanism for photoprotection of plants *in vivo* (Horton *et al.*, 1994).

The processes which lead to the formation of qE, causing the non-radiative dissipation of excess light energy (Ruban *et al.* 1994), are accompanied by changes in the pigment bed of LHC of PSII. The mechanism of qE is controlled by the trans-thylakoid membrane pH gradient ($\Delta$pH) and by the state of pigments involved in the xanthophyll cycle (Horton *et al.*, 1994; Gilmore, 1997). Acidification of the thylakoid lumen is due to proton translocation during electron transport and by release of protons during water splitting at the oxygen evolving complex. This acidification leads to the reversible de-epoxidation of violaxanthin to zeaxanthin through the enzyme violaxanthin de-epoxidase (VDE). Others authors suggest that the lumen pH determines the availability of violaxanthin (Pfündel *et al.*, 1994) while the pH of localised proton domains inside the membrane regulates the activity of VDE (Mohanty and Yamamoto, 1996). There are several possible roles for zeaxanthin in the induction of qE. First, zeaxanthin may directly accept, and quench, the singlet excited state of chlorophyll (Demmig-Adams and Adams, 1996). Second, the xanthophylls may be a factor in controlling the structure of LHCII (Horton *et al.*, 1996). A third possibility is that the xanthophylls may have a structural role in controlling the structure of the thylakoid membrane (Havaux and Tardy, 1995).

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The main goal of this study was to use triazine-resistant and susceptible biotypes of *Chenopodium album* to determine the relationship between herbicide-resistance and the sensitivity to photoinhibition. Earlier research has shown that *in vitro* chloroplasts isolated from R and S plants, show no difference in sensitivity to photoinhibition. However, *in vivo* R has a significantly lower Fv/Fm value after a photoinhibitory treatment than S. This result is especially so, when the plants have been grown at high irradiance (Chapter 3). The higher sensitivity of R to photoinhibition *in vivo* compared to S may be due to a difference in energy-dissipative mechanisms which can be photoprotective (Chapter 4).

In this study, the two biotypes were subjected to photoinhibitory irradiance after which photochemical and non-photochemical quenching was measured. In addition, the different components of non-photochemical quenching during the 'dark' relaxation were analysed. It was observed that the qP was lower in the R biotype than in the S plants. Among the components of qN, the qE was especially lower in the R biotype. It has been concluded that the enhanced sensitivity of the R plants to photoinhibition is related with shade-type characteristics, a lower rate of electron flow at the acceptor side of PSII and a lower proton gradient across the thylakoid membrane of the resistant plants.

**Material and methods**

*Plant material*

The origin and the growth conditions of the *Chenopodium album* L. plants used were as described in Jansen *et al.* (1986). Three weeks after germination, the plants were placed in a mixture of black soil and fine quartz sand (4:1) and transferred to other growth chambers. All the plants were grown under the same conditions: at a controlled temperature of 20°C, a light period of 16 h per day and a relative humidity of approximately 70%. The plants were grown under an irradiance of about 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR). As the light source TL-tubes (Philips) were used, specifically TLD 58 W/84.

*Measurement of fluorescence*

Chlorophyll *a* fluorescence was measured *in vivo* using whole leaves of both biotypes of *C. album*. The leaves were dark-adapted for at least 10 min. Measurements of modulated

chlorophyll fluorescence emission from the upper surface of the leaf were made using a pulse amplitude modulated fluorometer (PAM-101, H. Walz, Effeltrich, Germany). All measurements were carried out at 20 °C in an atmosphere of ± 20 % $O_2$. The actinic irradiance was the same for both biotypes, approximately 680 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (KL 1500, Schott). Calculations of the Chl fluorescence quenching parameters qP and qN were made according to Van Kooten and Snel (1990). $\phi_P$ was calculated as (Fm' - F)/Fm', while the activity of PSII electron transport (J) was calculated from fluorescence parameters using the method of Snel *et al.* (1991). All parameters were determined at steady state. The relaxation kinetics of the recovery of the different qN components (qE, qT, qI) in the dark were determined by applying the relationship (1-qN) = ((1-qNf) x (1-qNm) x (1-qNs) (Walters and Horton, 1991). The indices f, m and s refer to the fast, middle and slow phases respectively of qN relaxation in the dark (Lokstein *et al.*, 1993).

### *Photoinhibitory treatment*

Photoinhibitory treatments were carried out in a controlled environment. Several upper leaves of both R and S were harvested and put in a Petri dish filled with water. The central part of the leaf was exposed to photoinhibition light (± 4000 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Petioles of the leaves were held under water to enable the leaves to take up water during PIT. The duration of the PIT was varied to induce different levels of photodamage, indicated by the Fv/Fm values.

A few precautions were taken to prevent excessive transpiration of the leaf and to keep the leaf temperature at a constant level. The leaves were able to take up water through the petioles during the PIT and a flow of moisturised air was led along the upper surface of the leaf which was exposed to the photoinhibitory irradiance.

### Results

In triazine-resistant plants more reduction of $Q_A$ by light is due to the slower electron flow between $Q_A$ and $Q_B$. Thus, under non-photoinhibitory conditions (Fv/Fm is about 0.81), R has a much lower qP than S (see Fig. 5.1). During a photoinhibitory treatment (PIT), qP decreases in R at an earlier stage of photoinhibitory damage than in S; the latter appears to have

a kind of lag phase before the decrease of qP sets in (Fv/Fm < 0.7). At each level of photoinhibitory damage, R has a significantly lower qP than S (Fig. 5.1).
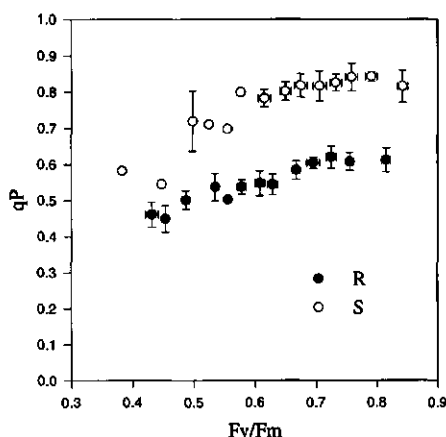


Fig. 5.1. Effect of PIT on the photochemical quenching (qP) of leaves of R and S of *Chenopodium album*. qP is shown as a function of the level of photoinhibitory damage, expressed as Fv/Fm. The bars in the figures indicate ± SD (n=2-32); SD bars not showing are within the pointmarkers.
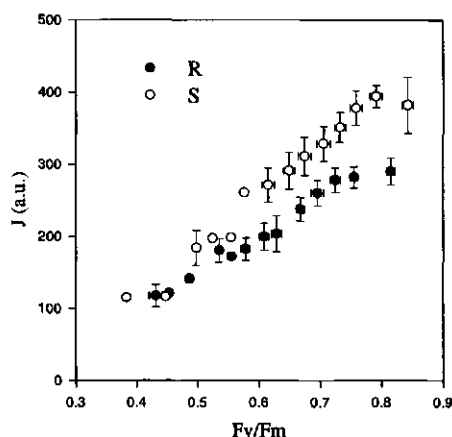
Fig. 5.2. Effect of PIT on the PSII electron transport (J) of leaves of R and S of *C. album*. J is shown as function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.

The two biotypes do not differ much in the dependence of J on PIT. However, the total decrease of J during the PIT (0.4 < Fv/Fm < 0.8) is significantly higher in S than in R (Fig. 5.2). Because of its mutation and in the absence of PIT, R has a reduced photochemical activity which leads to a lower J than in S. However, at increasing levels of photoinhibitory damage, the differences in J between R and S become less pronounced (Fig. 5.2).

The total amount of PSII reaction centers includes closed and open reaction centers. The fraction of open reaction centers is indicated by qP (Fig. 5.1) and the fraction of closed reaction centers by 1-qP (Fig. 5.3). Open reaction centers are capable of photochemical activity, while closed reaction centers can be active (but non-reducing) or inactivated (photoinhibited). The higher fraction of open reaction centers in S at similar PIT (qP in Fig. 5.1) causes a higher J in S compared to R (Fig. 5.2). Even at an

extreme high level of photoinhibitory damage (Fv/Fm = ± 0.4) both biotypes appear to have open reaction centers with photochemical activity (see Fig. 5.2).
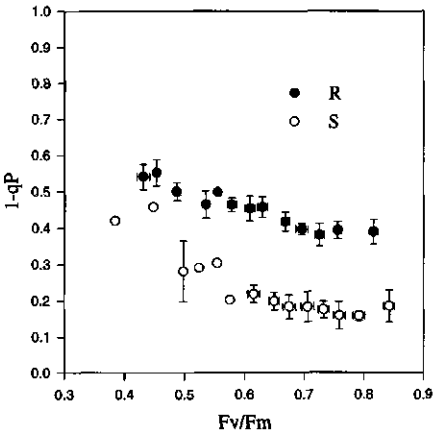


Fig. 5.3. Effect of PIT on the fraction of closed reaction centers (1-qP) of leaves of R and S of *C. album*. (1-qP) is shown as function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.
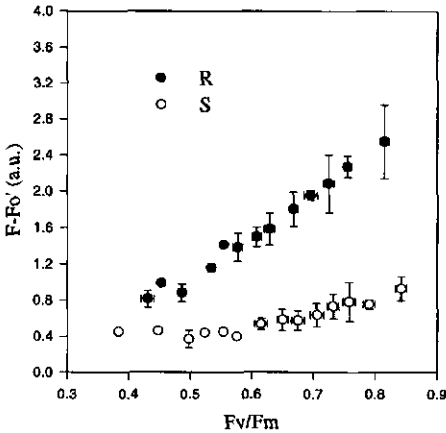
Fig. 5.4. Effect of PIT on the fluorescence level of closed reaction centers (F-Fo') of leaves of R and S of *C. album*. (F-Fo') is shown as function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.

Under non-photoinhibitory conditions, R has a higher fraction of closed reaction centers than S even during PIT (Fig. 5.3). At the highest levels of photoinhibitory damage (Fv/Fm < 0.5) the steady state level of fluorescence is independent of PIT in both biotypes (Fig. 5.4). This result indicates that the fraction of closed reaction centers then only consists of inactive reaction centers and shows no longer an increase in spite of increasing photoinhibitory damage (Fig. 5.3). In the case of R, the fluorescence level of these inactive centers is approximately two fold higher than in S (Fig. 5.4). This level is confirmed by (1-qP) under non-photoinhibitory conditions (Fig. 5.3) which shows that in the absence of PIT, R has twice as many closed reaction centers as S. Under these conditions (no PIT, low qN), the closed reactions consist mainly out of inactive centers, which are less sensitive to PIT, but still emit variable fluorescence (Schansker, 1996).

According to Laible *et al.* (1995), qP is underestimated due to the presence of inactive (non-$Q_B$ reducing) centers which do not participate in electron transport or oxygen evolution. The fraction of active reaction centers decreases during PIT by which the effect of the inactive centers on the calculation of qP will increase. Therefore it is necessary to correct for these inactive reaction centers. After a strong PIT, the fluorescence level in R from the fraction of inactive centers is about 6.5% of the Fv of a control leaf and 3.5% of the Fv of a control leaf in S (data not shown). By subtracting these fluorescence levels from the measured (Fm'-Fo') value (fluorescence emitted by all reaction centers), only the active centers are taken in account (Schansker, 1996). This calculation then gives a corrected value of qP (qP = (Fm'-F)/(Fm'-Fo')). In the case of R, the qP (corrected) does not decrease during PIT, but even shows a steep increase at a high level of photoinhibitory damage (Fv/Fm <0.6). The qP of S is not affected greatly by PIT (Fig. 5.5).


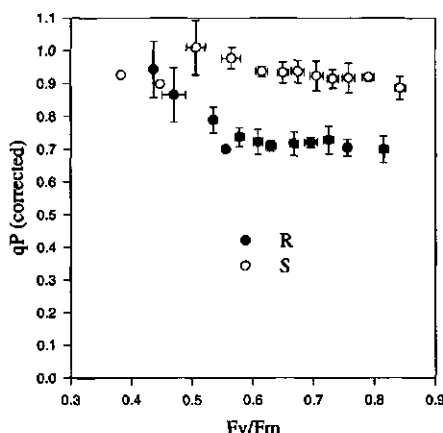
Fig. 5.5. Effect of PIT on the qP (corrected) of leaves of R and S of *C. album*. qP (corrected) is corrected for the fraction of inactive centers and is shown as function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.

When looking at the non-photochemical quenching (qN), before and after a period of photoinhibitory treatment (PIT), it appears that R has a lower capacity of non-radiative energy dissipation (Fig. 5.6). The differences between the two biotypes become smaller at a higher level of photoinhibitory damage (Fv/Fm < 0.6). The contribution of each component of qN (qE, qT, qN) to the amount of non-radiative energy dissipation of R and S in *C. album* during

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PIT was analysed. The "high-energy state" quenching in R, indicated by qE, has a lower value than in the S-type leaves (Fig. 5.7). This component of qN is a key factor in the non-radiative dissipation of excess energy and thus is one of the most important mechanisms of photoprotection. Control measurements (Fv/Fm = ± 0.81) have shown that in R the level of qE is approximately 0.1 and is always lower by a factor of about two compared to the S plants (Fig. 5.7). During photoinhibitory treatment (PIT) the change of qE in both R and S follows a modulating pattern with (for S) qE values between 0.3 and 0.15.
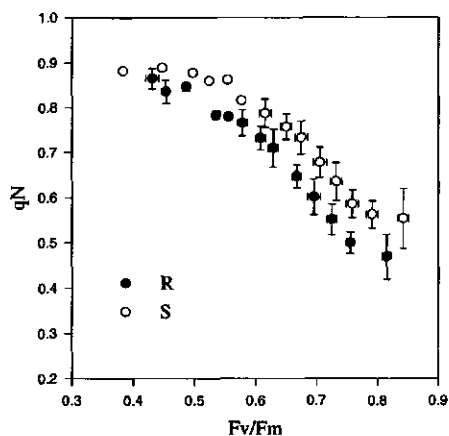


Fig. 5.6. Effect of PIT on the non photochemical quenching (qN) of leaves of R and S of *C. album*. qN is shown as a function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.
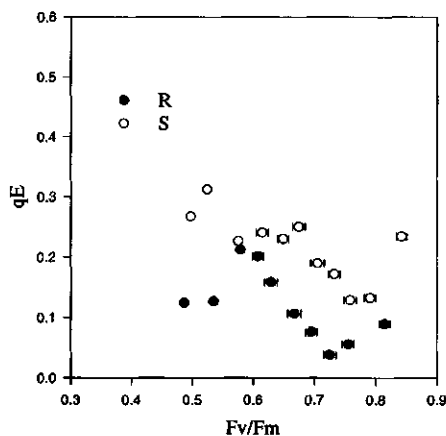
Fig. 5.7. Effect of PIT on the "high-energy state" quenching (qE) of leaves of R and S of *C. album*. qE is shown as a function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.

State 1 - state 2 transitions are caused by redox-controlled phosphorylation of LHCII (Allen, 1992). The phosphokinase is inhibited by PIT (Schuster *et al.*, 1986). Therefore, it may be expected that the fluorescence quenching due to state transitions, qT, may be influenced by photoinhibition. As illustrated in Fig. 5.8, qT is increased when a high level of photoinhibitory damage is reached (Fv/Fm < 0.55). qT does not change much with PIT for Fv/Fm > 0.55 and the two biotypes do not differ much in their magnitude of qT (Fig. 5.8).
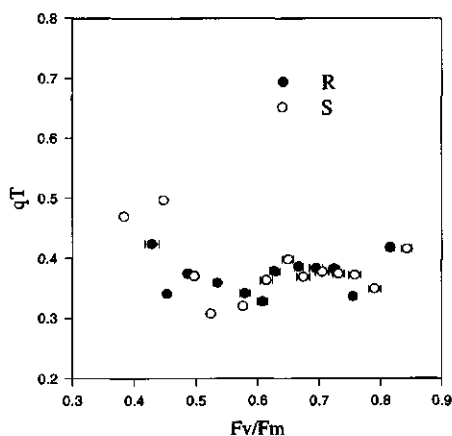
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Fig. 5.8. Effect of PIT on the quenching due to state-transitions (qT) of leaves of R and S of *C. album*. qT is shown as a function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.
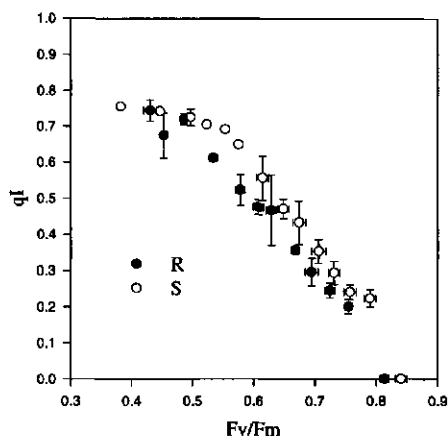
Fig. 5.9. Effect of PIT on the quenching due to photoinhibition (qI) of leaves of R and S of *C. album*. qI is shown as a function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.

During a photoinhibitory treatment, fluorescence quenching due to photoinhibition, qI, is induced. In the two biotypes, qI increases with increasing photoinhibitory damage (Fig. 5.9). However at similar level of qI, R has more photoinhibitory damage (lower Fv/Fm value) than S. The half time of the back relaxation of qI ($t_{1/2}$ of qI) shows a dramatic change at a level of photoinhibitory damage, corresponding with Fv/Fm being smaller than 0.55. At values of Fv/Fm $\leq$ 0.55, the half time of qI increases substantially for both biotypes (Fig. 5.10). This drastic increase in half time indicates that the repair of D1 protein becomes slower when photoinhibitory damages proceeds.

The variation in the absorbance cross section of LHCII is indicated by the level of Fo. R has a larger LHCII complex than S, leading to a higher level of Fo (data not shown). Changes in Fo quenching (q0) can be caused by state transitions, changes in the optical density and conformational changes in the pigment bed during the induction of qE (aggregation). q0 increases with increasing PIT (decrease in Fv/Fm). The effect becomes pronounced at Fv/Fm < 0.75 (Fig. 5.11). After PIT, the level of q0 is higher in R than in S, which is most likely due to the persistent higher Fo (larger peripheral LHCII) in R.

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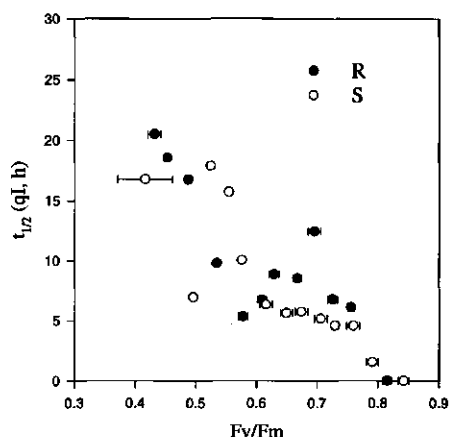
Fig. 5.10. Effect of PIT on the half time ($t_{1/2}$, h) of relaxation of qI of leaves of R and S of *C. album*. The half time of the relaxation of qI was derived from the relaxation kinetics of qN in the dark. $t_{1/2}$ of qI is shown as a function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend of Figure 5.1.
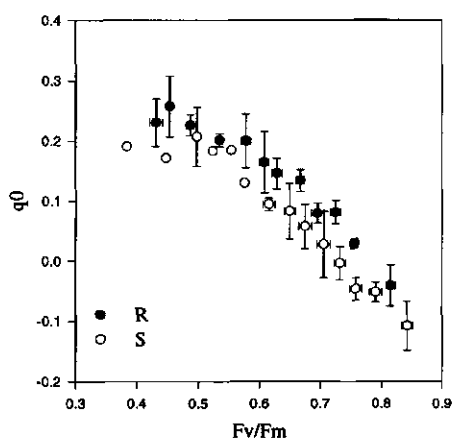


Fig. 5.11. Effect of PIT on the quenching of Fo (q0) of leaves of R and S of *C. album*. q0 is shown as function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend Fig. 5.1.

Fig. 5.12. Change in relative fluorescence change (RFC) of leaves of R and S of *C. album* during PIT. RFC is shown as function of the level of photoinhibitory damage, expressed as Fv/Fm. For further details see legend Fig. 5.1.

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The relative fluorescence change of chlorophyll (RFC = (F-Fo)/Fo) can be used to estimate the photosynthetic performance of plants (Snel *et al.*, 1994). This fluorescence parameter is a normalised standard, which represents the number of closed reaction cente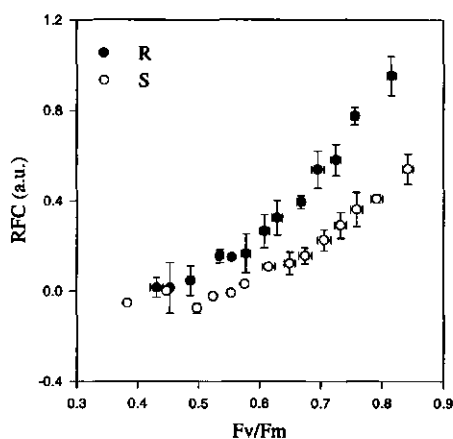rs. The higher level of RFC in R would indicate more closed reaction centers than in S leading to a higher level of fluorescence (Fig. 5.12). In both cases, the decrease of RFC is caused by enhancement of the Fo level and decrease of the F level (Fs).

**Discussion**

In triazine-resistant plants the electron flow rate between $Q_A$ and $Q_B$ has been found to be about three times lower than in susceptible ones (Jansen and Pfister, 1990). The reduced electron flow in triazine-resistant *Chenopodium album* is confirmed here (Fig. 5.2). In addition, although *Chenopodium album* is an extreme sun-type plant (Johnson *et al.*, 1994), the triazine-resistant biotype has shade-type chloroplasts (Vaughn, 1986).

The lower electron flow rate at the reducing side of PSII causes a lower qP in R plants (Fig. 5.2). During increasing photoinhibitory damage, both biotypes show a lag phase in decrease of qP, which sets in at a value of Fv/Fm of 0.7. The lower qP in the R plants is not only caused by the lower electron flow rate at the reducing side of PSII, but also by one of the shade-type characteristics of the R biotype: a larger LHCII (Vaughn, 1986). This shade-type characteristic causes a greater flow of photons to PSII (excitation pressure), inducing more closed reaction centers and less open reaction centers in R plants. The much lower qP in R plants gives less photoprotection in case of high irradiation levels.

The two biotypes do not only differ in the fraction of open active centers (Fig. 5.1), they show also a significant difference in the fraction of closed reaction centers (Fig. 5.3). The latter result is also a consequence of the inhibited electron flow rate between $Q_A$ and $Q_B$ (Fig. 5.2; Jansen *et al.*, 1986) in R plants and their larger LHCII. Both effects cooperate to cause a higher reduction level of PSII reaction centers. The higher PSII electron transport rate in S (Fig. 5.2) which corresponds with a larger fraction of open PSII centers (Fig. 5.1) appears to indicate that the reactions centers of both biotypes do not differ much in photochemical activity. This result leads to the conclusion that the decrease of qP during PIT is caused by a decrease in the fraction of open reaction centers and not by a reduction of photochemical activity per open reaction center during PIT.

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The seeming decrease in qP with PIT is due to the existence of inactive centers (Schansker, 1996) which causes at each PIT an underestimation of active centers relative to those that have become inactivated by PIT. Correction for these inactive centers completely changes the dependence of qP on PIT. Instead of a decrease of qP observed in Fig. 5.1, the corrected qP remains stable in the case of the S biotype (Fig. 5.5) and even increases at a Fv/Fm value below 0.6 in the case of R. This increase of qP, after high photoinhibitory damage, could be explained by a lower activity of PSII centers that have to supply electrons to PSI, causing a higher reoxidation level of $Q_A$, resulting in a higher qP. In spite of a larger fraction of inactive centers (Figs. 5.3-5.4), R is not able to form enough dissipative non-reducing centers, because the formation of effective dissipative centers requires sufficient acidification of the thylakoid lumen during light stress (Park *et al.*, 1996b).

In confirmation of earlier results, this study also fond a lower qN for the R biotype (Fig. 5.6). The different components of this non-photochemical quenching: qE, qT and qI were analysed. Under non-photoinhibitory conditions, Fv/Fm is approximately 0.8, R has a significant lower qE than S (Fig. 5.7). This result can be explained by the observation that R chloroplast membranes have a lower rate of PSII electron transport (Fig. 5.2) and less photosynthetic control than S (Rashid and Van Rensen, 1987). The first phenomenon leads to a slower acidification of the lumen via proton translocation across the thylakoid membrane accompanied with a lower production of protons from the water splitting complex. The second phenomenon indicates that the thylakoid is more permeable for protons. Both situations lead to less proton accumulation in the lumen and to a lower proton gradient which explain the lower qE in the R biotype.

The shade-type characteristic of R, reflected by the larger absorbance cross section of LHCII (Vaughn, 1986), would lead to a higher level of qE because this fluorescence quenching originates from the antenna pigment bed. However, one cannot easily determine from which part of the LHCII complex (minor, peripheral) the formation of qE arises (Dau, 1994). In the case of the R plants, it is conceivable that the major increase in LHCII complex is determined by the amount of peripheral LHCII not necessarily leading to an increase of qE. This phenomenon may also explain why, in spite of a higher amount of photoconvertible zeaxanthin (Chapter 4), R has still a lower qE under non-photoinhibitory conditions. If zeaxanthin is, for a major part, linked to the peripheral LHCII, there may be less contribution to the formation of qE under phosphorylating conditions. In the latter case

the peripheral LHCII becomes detached from LHCII complex in the state 1 - state 2 transition. Other research has also indicated that qE is apparently independent of zeaxanthin formation, especially in *Chenopodium album*, despite the fact that the xanthophyll cycle is active (Johnson *et al.*, 1994).

During increasing photoinhibitory damage, qE follows a modulating pattern (Fig. 5.7). After an initial decrease of qE at low photoinhibitory damage, there is an increase in qE below Fv/Fm of 0.75. Latter increase in qE could be due to inhibition of the protein kinase by photoinhibitory damage (Schuster *et al.*, 1986), leading to more dephosphorylation of LCHII (Fig. 5.8) and due to a larger antenna size, inducing a higher level of qE. At high photoinhibitory damage levels (Fv/Fm < 0.6) the qE decreases again in both biotypes. This may have several causes: a decrease in active reaction centers leading to less electron flow and less protons being deposited in the lumen (Schansker, 1996), increased membrane leakage leading to enhanced dissipation of protons from the lumen and/or a smaller ΔpH across the membrane.

According to most literature, qE gives photoprotection through non-radiative dissipation in the antenna pigment bed (Horton *et al.*, 1994). Hence, the lower qE is one of the main reasons why R is more sensitive to PIT *in vivo* than S. It was also recently reported by Darkó *et al.* (1996) that in triazine-resistant biotypes of *Conyza canadensis* qE was strongly reduced.

The quenching induced by state transitions, qT (Fig. 5.8), was approximately the same for R and S with a similar dependence on PIT. Although R and S do not differ in qT amplitude, the larger half time of qT in R (data not shown) could indicate that in resistant plants LHCII remains in a phosphorylated state for a longer period of time. Former process could be due to a slower dephosphorylation of LHCII (due to less activity of phosphatase) or caused by lower ΔpH in R via reduced electron flow rate (Fernyhough *et al.*, 1984).

The two biotypes show no difference in the kinetics of the increase of the qI, the quenching of fluorescence by photoinhibition, during increasing photoinhibitory damage (Fig. 5.9). The lower Fv/Fm value of R at the same level of qI may be caused by a lower amount of the D1 protein in the PSII reaction center of the R biotype. The D1 protein is the primary target of photoinhibition and in R, the D1 degradation may exceed the resynthesis of D1 already at a low level of photoinhibitory damage. ATP formation, necessary for the D1 resynthesis (Critchley and Russel, 1994), could be limited by inhibition of linear electron transport during PIT causing a lower D1 recovery. The latter process could lead to a strong increase of the half time of the dark relaxation of qI (Fig. 5.10). The R thylakoids have less

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CF1 (Vaughn, 1986), hence even under low photoinhibitory conditions R has less ATP available for D1 resynthesis. These events cause a higher half time of qI dark relaxation. At a very high level of photoinhibitory damage (Fv/Fm < 0.55), ATP availability becomes severly limiting and as a consequence the half time of qI in both biotypes goes to extreme values (Fig. 5.10). In addition to the inhibition of electron transport, the process of membrane leakage at a later stage of photoinhibitory damage, dissipating the ΔpH necessary for the functioning of ATPase, could also limit the amount of available ATP.

Quenching of Fo (q0) can result from the induction of qE (Ruban and Horton, 1994), chloroplast movement (Brugnoli and Björkman, 1992) or optical changes in the pigment bed. A difference between R and S in q0 is visible already at low levels of photoinhibitory damage (Fig. 5.11), when only low levels of qE are formed. This would indicate that the process of the induction of q0 depends on more than one mechanism. In the case of R it is possibly due to an altered LHC complex. In addition, other optical properties or enzymatic processes may add to an increased q0.

The relative fluorescence change (RFC) is a derivative of the RFD (relative fluorescence decrease) (Snel et al., 1994) and its light dependent response can give relevant physiological information about the state of the plant. A higher reduction level of $Q_A$, in R due to its mutation and possible higher excitation pressure (due to larger LHCII), are the main reasons why R has a higher fraction of closed reaction centers resulting in more fluorescence and a larger RFC compared to S (Fig. 5.12). The decrease of RFC during PIT in both biotypes is mainly due to increase of q0 during PIT. High-light induced chloroplast movement, changing the absorbance of the leaf, could induce strong Fo quenching (Brugnoli and Björkman, 1992). RFC is always higher in R than in S. Different processes could explain the higher RFC in R plants: light scattering, chloroplast movement, induction of qE (S > R), aggregation of LHCII or other structural changes in the antenna pigment bed.

Sensitivity towards photoinhibition can also be expressed by an index representing an intrinsic ability of PSII to balance photochemical and non-photochemical quenching, defined by the quotient (1-qP)/qN (Shen et al., 1996). During PIT, R experiences a higher excitation pressure (1-qP, fraction of closed reaction centers) and has less capacity for non-photochemical quenching (qN), resulting in a higher sensitivity to photoinhibition compared with the S plants. The R biotype appears to be less capable to balance its dissipative capacity (qP, qN) to compensate for excess light energy (Park et al., 1996a). This imbalance in the R

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biotype can be caused by one of its shade characteristics, the larger LHCII complex. The larger LHCII complex enhances the excitation pressure on PSII resulting in a higher fraction of closed reaction centers (1-qP), in addition to the reduced electron flow rate between $Q_A$ and $Q_B$ (lower qP).

In summary, triazine-resistant plants have a lower qP than the S biotype. This is caused by a slower electron flow activity of PSII and a larger LHCII complex. Furthermore, R has a lower non-photochemical quenching (qN) mainly caused by a lower qE. Thus R is less able to dissipate excess light energy in the antenna pigment bed of LHCII. The reduced electron transport activity of PSII in R, in combination with less photosynthetic control in the thylakoid membrane, causes a slower build up of $\Delta$pH across the membrane inducing a lower qE. In conclusion, the lower energy dissipation through qP and qE are the cause for the greater sensitivity to photoinhibition of resistant plants *in vivo*.

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**CHAPTER 6**

# ROLE OF ENERGY DISSIPATIVE PATHWAYS IN THE PHOTOPROTECTION OF TRIAZINE-RESISTANT AND SUSCEPTIBLE *CHENOPODIUM ALBUM* TO PHOTOINHIBITION

**Abstract**

Triazine-resistant (R) and susceptible (S) *Chenopodium album* plants differ in their sensitivity to photoinhibition. The difference is related to a lower level of photochemical (qP) and non-photochemical quenching (qN) in the R plants. Inhibitors of photoprotective pathways leading to qN were added to the leaves and the extent of photoprotection was studied by measuring the high- energy state quenching (qE), the quenching due to state transitions (qT) and other relevant fluorescence quenching parameters.

In the presence of dithiothreitol, which inhibits the formation of zeaxanthin (a carotenoid involved in the induction of qE), the R biotype showed no change in qE while S plants have a significant decrease of qE under non-photoinhibitory conditions. After a photoinhibitory treatment the level of photoinhibitory damage was higher in the presence of dithiothreitol, indicating that qE is involved in photoprotection. After infiltration of the leaves with sodium molybdate (inhibitor of phosphatase, which catalyses the dephosphorylation of LHCII complexes), the R biotype showed a relatively larger increase of qE than the S plants. Under photoinhibitory conditions, sodium molybdate seems to increase qT in both biotypes. Sodium fluoride seems to enhance qE in both biotypes, much like sodium molybdate, however sodium fluoride has only a positive effect on qT in the S leaves under photoinhibitory conditions.

In conclusion, qP as well as the components qE and qT are related to photoprotection. The activity of the processes associated with these three fluorescence parameters are apparently lower in the triazine-resistant plants.

**Introduction**

In triazine-resistant plants the D1 protein of photosystem II (PSII) is altered. The mutation involves a replacement of serine at site 264 by glycine (Hirschberg *et al.*, 1984). The result is a slower electron transport flow between $Q_A$ and $Q_B$. It was suggested that because of this reduction, triazine-resistant plants (R) are more sensitive to photoinhibition (Van Rensen *et al.*, 1990; Hart and Stemler, 1990). *In vitro*, the R biotype and the susceptible plants (S) show no difference in sensitivity to photoinhibition, however, recent reports confirm indeed

that *in vivo* R has a significantly lower Fv/Fm value than S after a photoinhibitory treatment (Chapter 3). The higher sensitivity of R to a photoinhibitory treatment (PIT) *in vivo* compared to S is related to a lower photochemical (qP) and non-photochemical (qN) quenching of chlorophyll *a* fluorescence (Chapter 4; Chapter 5). While the lower qP is caused by the lower activity of PSII in the R biotype, the lower qN is probably due to a lower capacity of the high energy-state quenching (qE). This qE is an important photoprotecting mechanism against photoinhibition *in vivo*.

Different energy dissipative mechanisms can be used by the plant to protect itself against excess light energy. These mechanisms enable the plant to respond within a short timescale (ranging from minutes to hours) to an abundance of energy (Dau, 1994). At present, it is not well understood when and how these photoprotection mechanisms function.

Non-photochemical quenching (qN) of chlorophyll fluorescence consists of three components of which qE, high-energy state quenching, is considered to be the most important one. It is still a matter of debate which mechanism induces qE. Driving forces for this dissipative process of excess energy are probably the proton gradient ($\Delta$pH) and the xanthophyll carotenoid zeaxanthin. Quenching ascribed to qT is related to state I-state II transitions and is induced via (de)phosphorylation of LHCII. How these regulating processes interact to result in the thermal dissipation of excess light energy in the antenna pigment bed of PSII is still being investigated (Horton *et al.*, 1996). By adding inhibitors of different quenching mechanisms (qE, qT) to the leaves, insight is gained about the involvement of these processes in the protection of the plant against excess light energy.

Dithiothreitol (DTT) is a very potent inhibitor of the zeaxanthin formation. DTT acts by reducing the disulphide which is necessary for the activation of the enzyme violaxanthin de-epoxidase (VDE) and in turn VDE converts violaxanthin into zeaxanthin (Yamamoto and Kamite, 1972). DTT also has side-effects. Possible effects are on APO (ascorbate peroxidase; Neubauer and Yamamoto, 1992; Gilmore and Yamamoto, 1993) and on the enzyme ATPase (Junesch and Gräber, 1985), however these effects are not taken in consideration in this research.

By using sodium molybdate (NaMo) as an inhibitor of phosphatase activity, the dephosphorylation and reversion of LHCII back to PSII (State I) is blocked (Barnett *et al.*, 1980). Inhibition of dephosphorylation by NaMo is possibly caused by the binding of the NaMo to the phosphate groups at the receptor site.

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Sodium fluoride (NaF), like NaMo, also blocks the transition from State II back to State I by inhibiting the activity of the enzyme phosphatase (Allen, 1995). The effect of the addition of NaF (or NaMo) *in vivo* would very much depend on the extent of the dark adaptation of the leaves. If, for instance only part of LHCII would be dephosphorylated (dark adapted, State I), when NaF (or NaMo) is added, the precise effect of both inhibitors on the process of state transitions is uncertain.

We have studied the effects of these inhibitors mentioned above on fluorescence parameters, including non-photochemical as well as photochemical components such as qP and qN. The results lead to the conclusion that phosphorylation and dephosphorylation of LHCII (measured by qT), transformation of violaxanthin to zeaxanthin and the ΔpH (measured by qE) are involved in photoprotection. The high energy state quenching, qE, is an important photoprotection mechanism which is less active in resistant plants.

**Material and methods**

*Plant material*

The origin and the growth conditions of the *Chenopodium album L.* plants used were as described in Jansen *et al.*, (1986). Three weeks after germination, the plants were placed in a mixture of black soil and fine quartz sand (4:1) and transferred to other growth chambers. All plants were grown under the same conditions: at a controlled temperature of 20°C, the light period was 16 h per day and the relative humidity was about 70%. The plants were grown under an irradiance of about 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (PAR). As the light source, TL-tubes (Philips) were used: TLD 58 W/84.

*Measurement of fluorescence*

Chlorophyll *a* fluorescence was measured *in vivo*; whole leaves of both biotypes of *C. album* were used. The leaves were dark-adapted for at least 10 min. Measurements of modulated chlorophyll fluorescence emission from the upper surface of the leaf were made using a pulse amplitude modulated fluorometer (PAM-101, H. Walz, Effeltrich, Germany).

All measurements were carried out at about 20 °C. The actinic irradiance was approximately 680 $\mu$mol·m$^{-2}$·s$^{-1}$ (KL 1500, Schott). Calculations of the Chl fluorescence quenching parameters qP and qN were made according to Van Kooten and Snel (1990). $\phi_P$ was calculated as (Fm' - F)/Fm', and the rate of PSII electron transport (J) was calculated from fluorescence parameters using the method of Snel *et al.* (1991). The relaxation kinetics of the different qN components (qE, qT, qI), in the dark, were determined by applying the relationship (1-qN) = ((1-qNf) x (1-qNm) x (1-qNs) according to Walters and Horton (see also Chapter 5). The indices f, m and s refer to the fast, medium and slow/irreversible phases respectively of steady-state qN relaxation in the dark.

### *Photoinhibitory treatment*

Photoinhibitory treatments (PIT) were carried out in a controlled environment. Different upper leaves of both R and S were harvested and put in a Petri dish filled with water. The central part of the leaf was exposed to photoinhibition light (± 4000 $\mu$mol·m$^{-2}$·s$^{-1}$), during 30 min. Petioles of the leaves were held under water to enable the leaves to take up water during PIT.

Different chemicals affecting several protective mechanisms were added to the Petri dish. 20 mM NaMo or 20 mM NaF was added to inhibit the state transitions of LHCII (Allen, 1995; Barnett *et al.*, 1980), and 15 mM DTT was added to inhibit zeaxanthin formation (Neubauer, 1993). The uptake of the chemicals by the petioles was ensured by exposing them to the solutions for at least 40 min, while weak light (± 50 $\mu$mol·m$^{-2}$·s$^{-1}$) was given to the leaves to maintain transpiration.

### Results

In the previous chapter, it was reported that in triazine-resistant plants the non-photochemical quenching, qN, is lower than in the susceptible biotype. In this chapter, qN was analysed into its different components (qE, qT, qI) and the effects of inhibitors of the various processes leading to qE, qT and qI were studied. In addition, the effects of these inhibitors on other fluorescence parameters were measured.

DTT is known to block zeaxanthin formation through inhibition of VDE (Neubauer, 1993). Furthermore, it has an inhibitory effect on the activity of APO under $CO_2$-limiting conditions (Neubauer and Yamamoto, 1992). Infiltrating the leaves with DTT had no effect on the Fv/Fm value under non-photoinhibitory conditions. However, when present during PIT, DTT enhanced the effect of photoinhibition in both biotypes (Fig. 6.1), with the effect being more pronounced in R. Most remarkable was the effect of DTT on the induction of qE which is inhibited in the case of the S plants under non-photoinhibitory conditions (Fig. 6.1). In the presence of DTT, photoinhibition leads to a lower qE than without DTT. The difference of this effect between the two biotypes was not significant.
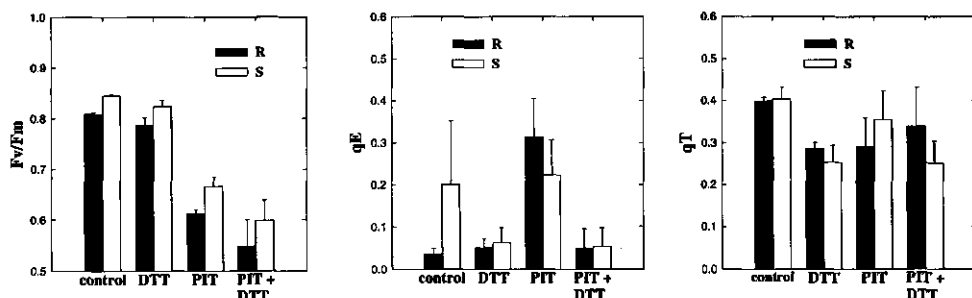


Fig. 6.1. The effect of 15 mM DTT in the absence and presence of photoinhibitory treatment (PIT) on the efficiency of open reaction centers of PSII (Fv/Fm), the 'high energy state quenching (qE) and on the quenching due to state transition (qT) of triazine-resistant (R) and susceptible (S) leaves of *Chenopodium album*.

Under non-photoinhibitory conditions, DTT reduces the level of qT (related to state transitions from State I to State II) in both R and S (Fig. 6.1). In the absence of DTT, a PIT leads to a slight decrease of qT. The presence of DTT during PIT does not significantly change the effect of PIT on qT. In comparison to Chapter 5, where qE was also studied, the qE in the plants used here is relatively small under non-photoinhibitory conditions. This low quenching might be due to the fact that plants of different batches have been used in the two experiments and therefore may be associated with a difference in state and condition of the leaves.

The two biotypes differ in their response of q0, quenching of Fo, to DTT under non-photoinhibitory conditions. This result is illustrated by the fact that the R biotype shows a

decrease in the presence of DTT while S does not (Fig. 6.2). Both biotypes enhance their q0 after PIT; however, S shows a more significant decrease of this enhancement in presence of DTT than R (Fig. 6.2).
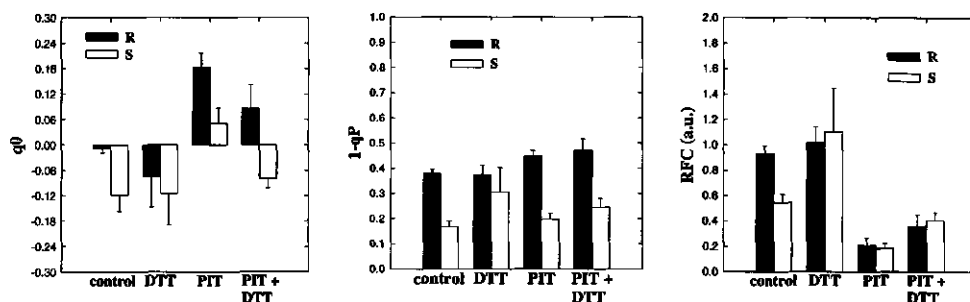


Fig. 6.2. The effect of 15 mM DTT in the absence and presence of PIT on the quenching of Fo (q0), the fraction of closed reaction centers (1-qP) and on the relative fluorescence change (RFC) of triazine-resistant (R) and susceptible (S) leaves of *C. album*.

From earlier research, it has been shown that R has a much higher fraction of closed reaction centers than the S plants (Chapter 4). The present results show the same (Fig. 6.2). After addition of DTT, the fraction of closed reaction centers in S is strongly increased while R showed no alteration. A PIT increased the fraction of closed reaction centers in both biotypes, however, more significantly in R. The extra addition of DTT did not significantly stimulate the effect of the PIT in either biotype (Fig. 6.2).

The R biotype has a higher relative fluorescence change (RFC), than S under non-photoinhibitory conditions. This result is probably caused by the presence of a more reduced state of $Q_A$, a lower qP and a lower qE in R (Chapter 4). The addition of DTT appears to enhance the RFC in S plants significantly under these conditions (Fig. 6.2). The inhibitory effect of PIT on RFC is reduced in the presence of DTT in both biotypes (Fig. 6.2).

Although the non-photochemical quenching components (qE, qT) are altered in the presence of DTT, it is remarkable that the photochemical components (qP (corrected), J and qP) are not altered significantly by DTT (Fig. 6.3). The presence of DTT before as well as after PIT had no influence, however, in the absence of PIT S showed a decrease of qP (corrected) in the presence of DTT.
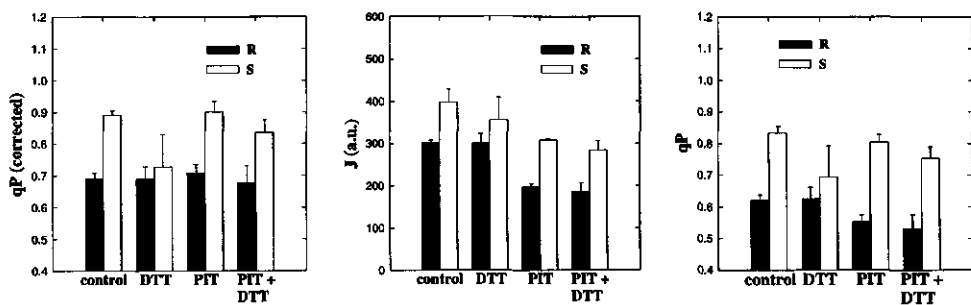
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Fig. 6.3. The effect of 15mM DTT in the absence and presence of PIT on the photochemical quenching corrected for inactive reaction centers (see qP(corrected), and see Chapter 5), the electron transport rate of PSII (J) and on the fraction of open reaction centers (qP) of triazine-resistant (R) and susceptible (S) leaves of *C. album*.
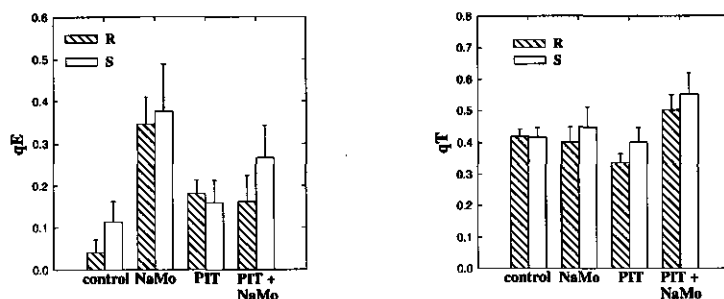


Fig. 6.4. The effect of 20 mM NaMo in the absence and presence of PIT on the 'high energy state quenching' (qE) and on the quenching due to state transition (qT) of triazine-resistant (R) and susceptible (S) leaves of *C. album*.

Under non-photoinhibitory conditions, NaMo had a substantial effect on qE in both R and S (Fig. 6.4), while the presence of NaMo during PIT tended only to increase qE in S. NaMo also affects the process of state transitions (qT) by enhancing qT in both biotypes under photoinhibitory conditions.

While DTT has no or little effect on the photochemical components (Fig. 6.3.), NaMo has an inhibitory effect on one of these components. The electron transport rate of PSII (J) is reduced by PIT and this effect is amplified by the presence of NaMo (Fig. 6.5). As an exception, qP (corrected) of the R plants seems to be slightly increased by NaMo before or after PIT (data not shown). In the absence of PIT, NaMo inhibited RFC in R plants, but had little to no effect on RFC in the S plants (Fig. 6.5). A PIT strongly reduces the amount of

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RFC and the addition of NaMo to the leaves amplifies this effect. Little or no effect of NaMo on the quenching of Fo (q0) was detected. Also, the fraction of closed reaction centers (1-qP) was not altered by NaMo before or after PIT. All these alterations by NaMo of both photochemical and non-photochemical components do not appear to influence the efficiency of the open reaction centers of PSII, Fv/Fm (data not shown).
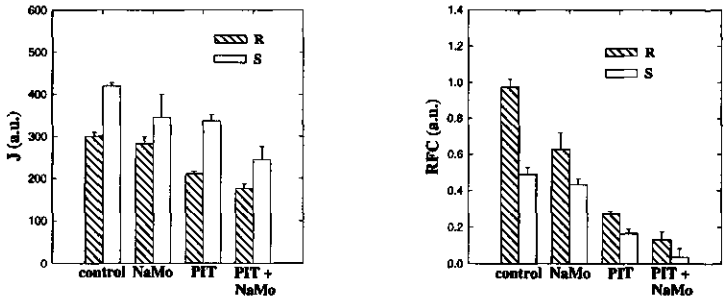


Fig. 6.5. The effect of 20 mM NaMo in the absence and presence of photoinhibitory treatment (PIT) on the electron transport rate of PSII (J) and on the relative fluorescence change (RFC) of triazine-resistant (R) and susceptible (S) leaves of *C. album*.
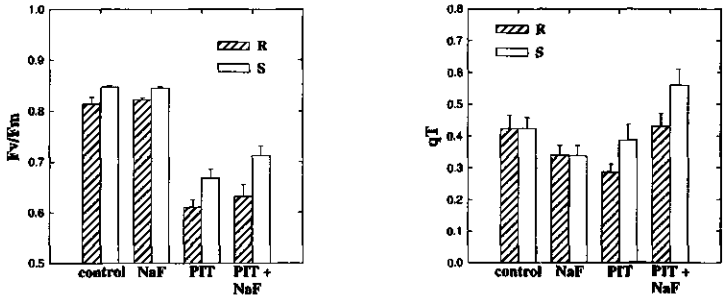


Fig. 6.6. The effect of 20 mM NaF in the absence and presence of PIT on the efficiency of open reaction centers of PSII (Fv/Fm) and on quenching due to state transitions (qT) of triazine-resistant (R) and susceptible (S) leaves of *C. album*.

NaF affects phosphatase, the enzyme which is involved in the dephosphorylation of LHCII complexes during the transition from State II to State I (Owens and Ohad, 1980). It appears that the presence of this chemical during PIT gives a reduction of the photoinhibitory effect on the Fv/Fm, but the effect is small (Fig. 6.6). The expected influence of NaF on state

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transitions is reflected by the level of qT and its dark relaxation time. However only under photoinhibitory conditions is there a tendency towards an enhancement of qT, in both S and R plants in comparison with the PIT (Fig. 6.6). Under non-photoinhibitory conditions there was only a negative response of q0 to the addition of NaF, leading to a reduced level of this quenching component (data not shown). All other measured parameters (photochemical, non-photochemical) were not significantly changed by the presence of NaF.

## Discussion

In chapter 5, it was shown that the high sensitivity for photoinhibition of triazine-resistant plants, compared to susceptible plants was related to a lower photochemical and non-photochemical quenching of fluorescence. The qN quenching includes several components (see chapter 5), each of which is driven by different mechanisms. The effects of chemicals, known to inhibit protective mechanisms (qP, qN) and its components were measured, including other fluorescence parameters.

The reducing compound dithiothreitol has little effect on the photochemical parameter qP and the fraction of open reaction centers, without or with photoinhibitory treatment. This result was found for both the R and the S biotype (Figs. 6.1 and 6.3), however, there is an important effect of DTT on the non-photochemical parameters. This chemical inhibits VDE which regulates zeaxanthin formation (Yamamoto and Kamite, 1972), and in turn zeaxanthin induces qE. In Fig. 6.1 the effects of DTT on qE are illustrated. The results inhibition of qE is more pronounced in S than in R, without PIT (Fig. 6.1), contradicts the earlier result that R has a higher amount of photoconvertible zeaxanthin than S (Chapter 4). A reason for this discrepancy may be that induction of qE does not depend on zeaxanthin via VDE alone, but also on the pH and the integrity of LHCII (Goss et al., 1995; Lokstein et al., 1993). Dithiothreitol also affects the formation of the ΔpH by inhibition of the APO activity, which mediates the linear electron flow necessary for lumen acidification (Neubauer and Yamamoto, 1992). The latter effect could imply that R is capable of dissipating the ΔpH at a lower rate than S, enabling R to maintain the qE for a longer period of time than S.

Zeaxanthin induces qE only when it is tightly bound to the lipid-protein matrix of the minor LHCII (Horton et al., 1996). The results in Chapter 4 imply that relatively more zeaxanthin is present in the R biotype as a mobile compound in the thylakoid membrane and

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not bound to the LHCII complex. This results explains the lower qE in R compared to S in spite of a higher zeaxanthin content. If this is the case, DTT would have less effect on qE in R plants under non-photoinhibitory conditions compared to S. When plants are exposed to high irradiance, possibly more zeaxanthin will be attached to LHCII to protect the plant against excess light energy. This effect is larger in R than in S. In the case that more zeaxanthin becomes attached to LCHII during a PIT, the presence of DTT during PIT will have more effect, resulting in a significant reduction of qE (Fig. 6.1).

The lower qT of both biotypes, in the presence of DTT under non-photoinhibitory conditions, is probably due to a side-effect of this inhibitor. It is known that DTT activates ATPase synthase (Junesch and Gräber, 1985). Due to enhanced ATP formation, less phosphate becomes available for the phosphorylation of LHCII. As a consequence less transition to State II would appear in the light, resulting in a lower qT (Fig. 6.1).

In the absence of photoinhibition, incubation with DTT leads to a stronger reduction of qE in S compared with R (Fig. 6.1). A lower qE gives less non-radiative dissipation of light energy, resulting in a higher level of fluorescence which is illustrated by a stronger increase of RFC in S plants in the presence of DTT (Fig. 6.2). In the case of the S biotype, qE is more strongly attenuated by the presence of DTT than in R. Former result implies that more photons will reach the PSII reactions centers leading to a significant increase of closed reactions under non-photoinhibitory conditions in the S plants in the presence of DTT (Fig. 6.2). The strong reduction of q0 in both biotypes after PIT in the presence of DTT (Fig. 6.2), also confirms that DTT has an effect on conformations in the antenna pigment bed via state transitions (phosphate availability) and ΔpH (ATPase modification). It is clear that DTT also has a significant impact on non-photochemical components (qE and qT), which are controlled by zeaxanthin formation and ΔpH. Furthermore DTT has much less an effect on the photochemical components (qP and J).

Sodium fluoride affects the process of state transitions by inhibiting phosphatase activity (Owens and Ohad, 1988), while sodium molybdate exerts its effect on phosphatase by the binding to the phosphate groups at the receptor site, thereby preventing dephosphorylation (Barnett et al., 1980). The reason why NaMo enhances the qE in R is not quite clear. It is possible that NaMo binds to the receptor site of the ATPase and blocks the phosphorylation of ADP to ATP. This action inhibits the dissipation of ΔpH, leading to more acidification of the lumen and to a higher level of qE (Fig. 6.4).

While control measurements show little or no effect of NaMo on qT, enhancement of qT in both biotypes after PIT in the presence of NaMo is clearly visible (Fig. 6.4). This result implies that enhancement of qT by inhibiting dephosphorylation of phosphorylated LHCII via NaMo is most effective when the phosphorylation of LHCII is blocked by PIT.

A lower RFC value could indicate an alteration in light absorbance by the leaf through the process of chloroplast movement (Burgnoli and Björkman, 1992). Recently, it has been shown that in shade plants chloroplast movement is more pronounced, and may decrease the absorption of light as photoprotection against high irradiance (Park et al., 1996). The R plant acts like a shade plant (Vaughn, 1986) and the higher level of chloroplast movement could explain the larger amount of light scattering which was observed earlier in R plants (Chapter 4). A shade plant such as the triazine-resistant C. album also has larger LHCII complexes. A larger absorbance cross section of LHCII can also be a disadvantage in situations of excess light energy, because a greater amount of photons will reach PSII causing more closed reaction centers. A higher fraction of closed reaction centers causes a decrease of qP and therefore dissipation of excess energy through photosynthetic pathways is reduced. This reduction enhances the sensitivity to photoinhibition of R compared to S.

Although sodium fluoride has no effect on the Fv/Fm value in the absence of photoinhibition, NaF seems to reduce the photoinhibitory damage (Fig. 6.6) by inhibiting dephosphorylation of phosphorylated LHCII. In the latter case, less photons will reach PSII because relatively more LHCII will be in State II (phosphorylated) reducing the absorbance cross section of PSII. A smaller LHCII reduces the chance of overexcitation of the reaction centers in case of excess light energy, hence less photoinhibitory damage. The effects of NaF on qT (Fig. 6.6) are comparable to what has been observed with NaMo (Fig. 6.4).

The present results confirm the conclusion drawn in the chapter 5 that not only qP but also qN is involved in photoprotection. Both components have a lower activity in the R biotype. The inhibitor studies reveal that the processes leading to the qT (phosphorylation and dephosphorylation of LHCII) and those leading to qE (violaxanthin to zeaxanthin conversion and the formation and dissipation of the ΔpH) are involved in photoprotection. The higher degree of photoinhibitory damage when PIT is given in the presence of DTT, confirms the earlier conclusion that qE is an important photoprotection mechanism which is less active in the resistant biotype as compared to the susceptible plants.

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# CHAPTER 7

## GENERAL DISCUSSION

Triazine-resistance in several weed species was discovered at the beginning of the seventies (Ryan, 1970). Ever since there has been a great deal of research to determine the differences and similarities in photosynthesis and energy dissipative pathways, between triazine-resistant (R) and susceptible (S) biotypes. The work presented in this PhD-thesis, concerning *Chenopodium album*, clearly shows that the R biotype has less tolerance than S plants under stress conditions. The R plants lack the ability to dissipate excess light energy efficiently, through photochemical and non-photochemical processes (Chapter 3; Chapter 4), in order to protect themselves against photoinhibitory damage. Specific differences between the two biotypes, which cause the higher sensitivity of R towards photoinhibition are discussed in this chapter.

**Growth competition**

When triazine-resistant plants were grown in growth chambers at moderate irradiance level, the maximum photosynthetic capacity (expressed as either carbon dioxide fixation or as oxygen evolution), was not much different from the wild (susceptible) biotype (Van Oorschot and Van Leeuwen, 1984; Jansen *et al.*, 1986). The research presented here, revealed that differences between R and S in growth performance and/or photosynthetic capacity depends on the conditions during the growth period (Chapter 3). The R biotype behaves like a shade-type plant that survives better under low light conditions (shade) (Van Rensen *et al.*, 1990). Under these conditions it can be just as competitive as the S biotype. This behaviour would indicate that under natural conditions, R would benefit more from shaded, densely grown places (*i.e.* maize field) while in the open field S would flourish because of its more sun-type characteristics (high photosynthetic capacity).

**In vivo and in vitro**

Measurements *in vivo* (Chapter 3-6) revealed that R and S differ in their ability to convert or dissipate excess light energy. The extent of the difference depends on the conditions during the growth period. High irradiance during growth results in a high photosynthetic activity of S, while R has more difficulty to cope with a high amount of excess light energy. It appears that the mutation which makes R resistant to the inhibitory action of

the herbicide atrazine, has a negative effect on the ability of R to protect itself against an abundance of light energy.

*In vitro* (Chapter 2) R and S are similar in their sensitivity towards photoinhibition. This result is caused by the absence or lower activity of the light-energy dissipative or -converting mechanisms that exert the deviation between the *in vivo* and *in vitro* experiments. It is possible that during the process of chloroplast isolation, several important defence mechanisms have been damaged or removed from the chloroplasts. Photorespiration, which can be protective against photoinhibition (Wu *et al.*, 1991; Heber *et al.*, 1996), is dependent on factors present in the stroma. Those stromal factors are diluted in suspension of broken chloroplasts, rendering this protective mechanism more or less inactive. Furthermore, the protection by energy quenching (qE) requires a proton gradient ($\Delta$pH) which regulates the formation of qE (Horton *et al.*, 1996). The use of broken chloroplast suspension increases the changes of membrane leakage, which reduces the formation $\Delta$pH and qE. This reduction makes the chloroplast more vulnerable to photoinhibitory damage. Zeaxanthin formation is also influenced by the $\Delta$pH. Less zeaxanthin formation resulting from additional membrane leakage could lower the photoprotective capacity of broken chloroplast suspension against photoinhibition.

## qP, photochemical quenching

Triazine-resistant plants have an impaired activity of PSII electron transport (ET in Fig. 7.1) which results in a higher reduction state of $Q_A$ in the light. A higher reduction level of $Q_A$ may be expected to cause less photochemical quenching (qP). When plants are grown at high and low irradiances (Chapter 4), it appears that plants of both biotypes grown at high light have a larger qP than the plants grown at low irradiance. Although the difference in qP between the two biotypes grown at low irradiance becomes smaller, the R biotype always has a lower qP than the S plants (Fig. 7.1). This difference in qP indicates a lower capacity to convert light energy via electron transport in the R biotype. Because of the lower activity of this photoprotective process, R plants are more sensitive to photoinhibition.

## Zeaxanthin formation

The R biotype was found to have a much higher formation of photoconvertible zeaxanthin after growth under high irradiance than the S biotype (Chapter 4). This result

could be related to a difference in activity of the violaxanthin de-epoxidase (VDE) which converts violaxanthin to zeaxanthin. This enzyme is dependent on the presence of the galactolipid monogalactocyldiacylglyceride (MGDG) for its activity (Yamamoto and Higashi, 1978). R has been found to have a higher amount of MGDG in its thylakoid membrane (Burke *et al.*, 1982). Therefore, it is possible that *in vivo* the conversion of violaxanthin to zeaxanthin is higher in R where VDE is in the vicinity of a higher concentration of MGDG (Rockholm and Yamamoto, 1996), leading to higher amounts of zeaxanthin. Former assumption would suggest that the formation of zeaxanthin not only depends on ΔpH (lumen, localised proton domains) but also on the structural aspects of the thylakoid membrane (lipids, protein-pigment complexes). A higher zeaxanthin content in the thylakoids of R plants compared to S ones could be endogenous to this biotype under certain growth conditions. Furthermore in the presence of zeaxanthin, light harvesting complexes become less stabile and more thermolabile (Tardy and Havaux, 1996a). According to this assumption, the function of the xanthophyll cycle lies in the dynamic aspects of violaxanthin transformation; zeaxanthin formation results in increased membrane viscosity and enhanced stability to heat stress (Tardy and Havaux, 1996b).

If zeaxanthin also acts as an accessory pigment by harvesting light in the vicinity of the LHC of PSII, it can increase the exciton pressure (1-qP) on PSII. This increase causes more reaction centers to close, which in turn lowers the photosynthetic capacity of R plants. On the other hand zeaxanthin can accept energy from triplet chlorophyll (Demmig-Adams and Adams, 1996) thereby reducing the formation of active oxygen species. In this case, zeaxanthin itself will act as a quencher of oxidative radicals species.

**LHCII, antenna complex of PSII**

Most of the changes related to the induction of qE are suggested to occur directly in or in the vicinity of LHCII (the antenna complex of PSII). The results in this work indicate not only that R and S differ in the structural arrangement of LHCII (aggregation, absorption cross section; Fig. 7.1), but also in optical properties such as scattering and chloroplast movement. This movement protects PSII from light stress (Park *et al.*, 1996). Shade plants show more chloroplast movement than sun-type plants. In R plants this process could lead to a higher level of q0 (Chapter 5), but also to more change in light scattering (Chapter 4).

## qE, high energy state quenching

In spite of its higher content of photoconvertible zeaxanthin (Chapter 4), R has a significant lower qE compared to S (Chapter 5). It is possible that in R plants a larger part of the xanthophyll cycle carotenoids is more loosely bound or is indirectly bound to the LHC pigment-proteins or to other proteins (Lee and Thornber, 1995). Less zeaxanthin would then be involved in the induction of qE (Fig. 7.1) or the regulation of structural arrangement in the LHCII (aggregation) in the R plants. Therefore, a higher zeaxanthin content does not necessarily lead to a higher formation of qE.

The question may be raised how important the difference in qE between R and S is, for the higher sensitivity to photoinhibition of R in cases of excess irradiance. *Chenopodium album* is as a typical sun plant that has a good photochemical activity for dissipating excess energy and little effort is put in non-photochemical quenching (Johnson *et al.*, 1994). qE in R plants becomes relatively more important as an additional protective mechanism than in S. This phenomenon is due to the significant lower photosynthetic capacity in R plants.

## Inactive PSII centers

Photoinhibition experiments have shown (Chapter 5) that the triazine-resistant plants have a significantly higher fraction of inactive reaction centers than the susceptible plants. These inactive centers behave similar to the $PSII_\beta$, as non-reducing centers (Chylla *et al.*, 1987). The inactive centers have smaller effective absorption cross section of LHCII (Van Rensen and Spätjens, 1987), have a slower turnover and are possibly located in the stroma region. It is not unlikely that a higher percentage of inactive centers in R contribute to a lower qE, through a slower electron flow rate to the plastoquinone pool (less lumen acidification) or through the presence of a smaller antenna pigment bed (less fluorescence). Although at a lower rate, inactive centers can transfer absorbed light energy to PSII. The absorbed energy enhances the excitation pressure, which in turn induces more closed reaction centers (Terao and Katoh, 1996).

## Photoinhibitory damage

The results presented in this thesis demonstrate that R and S of *Chenopodium album* differ in their regulation of photosynthesis and energy dissipative mechanisms in response to

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abiotic stress. The R plants do not only have an impaired PSII electron transport rate, but also a larger antenna complex, increasing its exciton pressure (1-qP) on PSII which causes more closed reaction centers and a lower photosynthetic activity.

To summarise the different energy converting and energy dissipating mechanisms of R and S which protect them against photoinhibition, the following model is suggested. The regulation of photosynthesis and energy dissipative mechanisms in triazine-resistant and susceptible *Chenopodium album* are considered in this model.
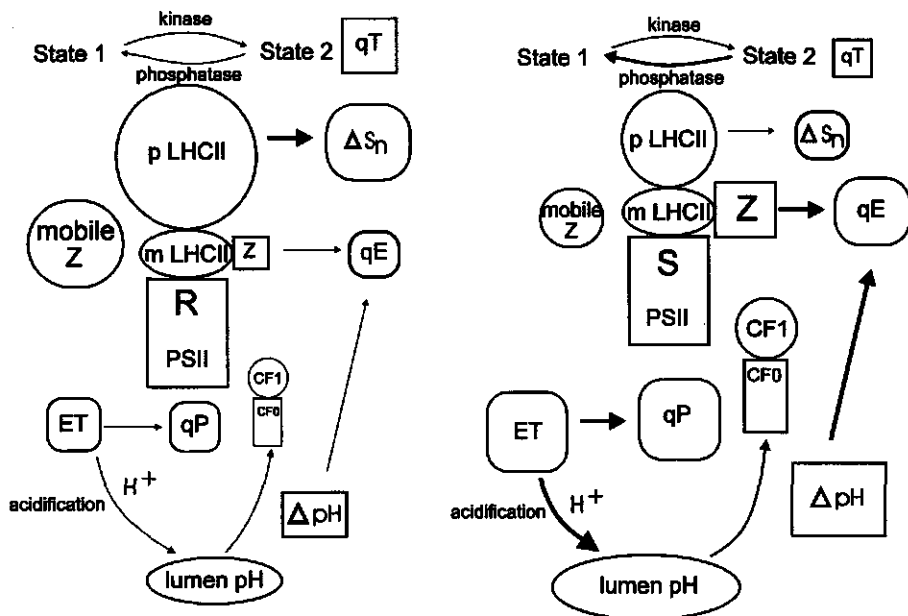


Fig. 7.1. Schematical display of the regulation of photosynthesis and energy dissipative mechanisms of triazine-resistant and susceptible *Chenopodium album*. The area of the closed geometrical forms represents the fraction size of the photosynthetic parameter and the thickness of the arrows indicates the conversion/induction rate of the different reactions. mLHCII: minor LHC of PSII; pLHCII: peripheral LHC of PSII.

## Concluding remarks

The major differences in photosynthesis and energy dissipation between triazine-resistant and susceptible *Chenopodium album* are the lower qP and qE in R plants. As a consequence R is less able to cope with situations of excess light energy, leading to more photoinhibitory damage of the photosynthetic apparatus compared to S.

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# Summary

As a consequence of the intensive use of herbicides during crop growth, many herbicide-resistant biotypes have evolved. One of the first examples is the resistance of *Chenopodium album* against triazine-type herbicides. About ten years after this discovery, it was observed that triazine-resistant plants (R) have a lower rate of electron flow at the acceptor side of photosystem II. Later, the chloroplasts of the resistant biotype were described as having shade-type characteristics. In addition, the R biotype was found to have an altered lipid composition of the thylakoid membrane, to be more sensitive to high temperature and the thylakoid membrane has a low affinity for bicarbonate. In the presence of high irradiance, R is retarded in growth and development compared to the susceptible (S) biotype and has a higher sensitivity to photoinhibition (this thesis). The goal of this work was to contribute to a better understanding of the mechanistic relationship between light stress and photosynthetic yield, *i.e.* biomass production.

In chapter 2 the effect of photoinhibition on electron transport and photophosphorylation in isolated chloroplasts is described. This research proved that photoinhibition causes a gradual uncoupling between electron transport and phosphorylation. This indicates that photoinhibition causes a proton leakiness of the thylakoid membrane. An important observation was that *in vitro* the two biotypes do not differ from each other in their sensitivity towards photoinhibition.

Because R and S do not differ much in their sensitivity to photoinhibition *in vitro*, research was performed for the *in vivo* situation with intact leaves (Chapter 3). When plants have been grown at low light irradiance, little or no differences in dry matter productions appear to exist between the two biotypes. However, growth at high light irradiance causes a significantly lower production of the resistant biotype. Fluoresence studies indicated that the lower productivity of the resistant plants is caused by a higher sensitivity to photoinhibition. The less significant differences *in vitro* in comparison to the *in vivo* situation are probably caused by the loss or lower activity of photoprotective mechanisms during the isolation of chloroplasts.

Further research into these protective mechanisms (Chapter 4) revealed that R shows more light-induced zeaxanthin formation and a larger change in light scattering than S, especially when grown at high irradiance. The difference in level of non-photochemical

quenching (qN) is more pronounced at low light irradiance. Photorespiration acts as an energy dissipative mechanism and appears to be more important in R than in S plants. In conclusion, the increased sensitivity to photoinhibition of resistant plants is not caused by a lower activity of the photoprotective pathways of zeaxanthin formation and photorespiration. The shade-type characteristics of the chloroplasts of the resistant plants are important for the greater sensitivity to photoinhibition.

Because qN includes several components, research has been performed to study the role of these components and the role of photochemical quenching (qP) in the differences between R and S in their sensitivity to photoinhibition (Chapter 5). The lower qP in the R plants is explained by a larger absorbance cross section of photosystem II (PSII) in the shade-type chloroplasts of the R plants, which enhances the odds of excitation of PSII. In combination with a reduced rate of electron flow at the reducing side of PSII, a higher excitation pressure causes an increase in the fraction of closed reaction centers. The observed lower qE in the R biotype is suggested to be due to a lower PSII electron flow rate and a lower photosynthetic control in R compared with that in S, leading to a smaller proton gradient across the thylakoid membrane. From these findings, it was concluded that the lower energy dissipation through qP and qE cause the greater sensitivity to photoinhibition of resistant plants *in vivo*.

The last topic of this research, was the study of the effect of several inhibitors of the different energy dissipative mechanisms (qE, qT) to examine of the contribution of these mechanisms to photoprotection (Chapter 6). Addition of these inhibitors was found to lead to increased photoinhibitory damage, especially in the R biotype.

In summary, triazine-resistant plants behave like shade-type plants and are adapted to low light irradiances (maize field, forests). The wild-type *Chenopodium*, a typical sun plant, has more tendency to grow in the open field (road-side, meadow). When R is exposed incidentally or permanently to high irradiance, it will suffer more photoinhibitory damage than the wild biotype (S). The R biotype will be retarded in growth and development due to lack of sufficient activity of photoprotective mechanisms and photosynthetic capacity.

# Samenvatting

Het intensief toepassen van chemische onkruidbestrijding bij de teelt van gewassen gedurende enkele decennia heeft geleid tot een groot aantal herbicide-resistente planten. Een van de eerste voorbeelden is de resistentie van *Chenopodium album* tegen triazine-type herbiciden. Ongeveer tien jaar na de ontdekking van deze resistentie werd waargenomen, dat de triazine-resistente planten (R) een lagere snelheid van het elektronentransport aan de reducerende zijde van fotosysteem II hebben. Enige tijd later werden de chloroplasten van het resistente biotype beschreven als zijnde schaduw-type chloroplasten. Bovendien bleek het R biotype een veranderde lipidensamenstelling van het thylakoidmembraan te hebben, gevoeliger te zijn voor hoge temperatuur en een lagere affiniteit te bezitten voor bicarbonaat. Bij hoge lichtintensiteit blijft R achter in groei en ontwikkeling in vergelijking met het wilde type (S) en heeft het een verhoogde gevoeligheid voor fotoinhibitie (dit proefschrift). De doelstelling van dit project was de mechanistische relaties tussen lichtstress en fotosyntheseopbrengst beter te leren kennen.

In hoofdstuk 2 wordt het effect van fotoinhibitie op elektronentransport en fotofosforylering in geïsoleerde chloroplasten beschreven. Uit dit onderzoek blijkt dat fotoinhibitie een langzame ontkoppeling veroorzaakt van het elektronentransport met de fosforylering. Hieruit blijkt dat fotoinhibitie een toenemende protonenlekkage veroorzaakt in het thylakoidmembraan. Een belangrijke waarneming is dat *in vitro* de twee biotypen (R en S) niet of weinig van elkaar verschillen in hun gevoeligheid voor fotoinhibitie.

Aangezien R en S *in vitro* weinig verschillen in fotoinhibitiegevoeligheid is er gekeken naar de situatie en effecten in intacte bladeren (hoofdstuk 3). Wanneer de planten bij lage lichtintensiteit worden opgekweekt blijken er weinig verschillen in droge stof productie tussen beide biotypen te bestaan, maar opkweek bij hoge lichtintensiteit geeft een significant lagere productie bij het resistente biotype. Uit fluorescentie metingen blijkt dat de lage productiviteit van de resistente planten wordt veroorzaakt door een hogere gevoeligheid voor fotoinhibitie. De weinig significante verschillen *in vitro* in vergelijking met de *in vivo* situatie worden mogelijk veroorzaakt door het verlies of verminderde activiteit van beschermingsmechanismen tijdens de isolatie van chloroplasten.

Nader onderzoek van deze beschermingsmechanismen (hoofdstuk 4) heeft uitgewezen dat vooral wanneer opgekweekt bij hoge lichtintensiteit, R, meer licht-geïnduceerde

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zeaxanthinevorming en lichtverstrooïng vertoont dan S, terwijl het verschil in niet-fotochemische fluorescentiedoving (qN) meer geaccentueerd is bij opgroei bij lage lichtintensiteit. Fotorespiratie blijkt als energiedissipatie-mechanisme in R veel belangrijker te zijn dan in S planten. Hieruit werd voorlopig geconcludeerd dat de verhoogde gevoeligheid van R voor fotoinhibitie niet veroorzaakt wordt door verminderde activiteit van zeaxanthinevorming en fotorespiratie. De schaduwachtige karakteristieken van de R chloroplasten spelen een rol bij de verhoogde gevoeligheid voor fotoinhibitie.

Aangezien de niet-fotochemische doving uit meerdere componenten bestaat is gekeken naar de rol van de componenten van qN en naar de rol van de fotochemische fluorescentiedoving (qP) bij R en S in de gevoeligheid voor fotoinhibitie. Dit werk is beschreven in hoofdstuk 5. De veel lagere qP bij R planten wordt verklaard door het grotere antennecomplex van fotosysteem II (PSII) in de schaduw-type chloroplasten van R, die de kans op excitatie van PSII verhogen en daarmee, in combinatie met de lagere snelheid van het PSII elektronentransport, het aantal gesloten reaktiecentra vergroten en het niveau van qP verlagen. De waargenomen lagere qE wordt mogelijk niet alleen veroorzaakt door een lagere PSII elektronentransportsnelheid, maar ook door minder fotosynthetische controle in R in vergelijking met S, wat leidt tot een lagere protonengradiënt over het thylakoïd membraan. Uit deze bevindingen wordt geconcludeerd dat de verminderde energiedissipatie via qP en qE de oorzaak zijn voor de verhoogde gevoeligheid van resistente planten tegen fotoinhibitie *in vivo*.

Tenslotte is er gekeken naar het effect van verschillende remstoffen op de verschillende beschermingsmechanismen van qN (qE, qT) en daarmee naar de gevoeligheid van R en S voor fotoinhibitie in de aanwezigheid van deze chemicaliën (hoofdstuk 6). Het toevoegen van deze remstoffen leidt voornamelijk in R tot meer fotoinhibitieschade.

Concluderend, blijkt het dat triazine-resistente planten als schaduwtype planten het best zijn aangepast aan lagere lichtintensiteiten (maisveld, bebosd gebied), terwijl de wild-type *Chenopodium*, typische zonneplanten, zich meer op hun gemak voelen in het open veld (berm, weiland). Wanneer het R biotype incidenteel of permanent aan hoge lichtintensiteiten wordt blootgesteld, zal het door gebrek aan voldoende beschermingsmechanismen en fotosynthesecapaciteit zich slechter handhaven dan de wild-type plant (S) en in groei en ontwikkeling achterblijven.

# List of Publications

1.  Curwiel, V.B., Schansker, G., De Vos, O.J. and Van Rensen, J.J.S. 1993. Comparison of photosynthetic activities in triazine-resistant and susceptible biotypes of *Chenopodium album*. Z. Naturforsch. **48c**, 278-282.

2.  Curwiel, V.B. and Van Rensen, J.J.S. 1993. Influence of photoinhibition on electron transport and photophosphorylation of isolated chloroplasts. Physiol. Plant. **89**, 97-102.

3.  Curwiel, V.B., Schansker, G., De Vos, O.J. and Van Rensen, J.J.S. 1994. Molecular mechanisms of photoinhibition, an abiotic stress factor limiting primary plant production. In: Proc. Int. Congress on Plant Production on the Threshold of a new Century, (P.C. Struik *et al.*, eds), pp. 423-425, Kluwer Academic Publishers, The Netherlands.

4.  Curwiel, V.B. and Van Rensen, J.J.S. 1995. Relation of fluorescence quenching and other energy dissipative pathways with photoinhibition in leaves of triazine-resistant and susceptible *Chenopodium album*. In: Proc. Xth Int. Photosynthesis Congress, (P. Mathis, ed.), pp. 247-250, Kluwer Academic Publishers, The Netherlands.

5.  Curwiel, V.B. and Van Rensen, J.J.S. 1996. Chlorophyll fluorescence quenching, zeaxanthin formation and light scattering in intact leaves of triazine-resistant and susceptible *Chenopodium album* plants. J. Photochem. Photobiol. B: Biology, **35**, 189-195.

# Curriculum vitae

Victor Bas Curwiel werd op 4 november 1963 geboren te Den Haag. Na het voltooien van de VWO studie op het Fioretti College te Lisse, begon hij in 1982 met een botanische analisten opleiding op de toenmalige STOVA te Wageningen. Tijdens deze opleiding werd er een afstudeervak uitgevoerd (bij dr. ir. A. Varga) op de vakgroep Plantenfysiologie. In 1986, na het beëindigen van deze analisten opleiding, werd er een aanvang gemaakt met de studie Landbouwplantenteelt op de toenmalige vakgroep Landbouwplantenteelt (thans Agronomie) aan de Landbouwuniversiteit te Wageningen. Tijdens de doctoraalfase waren de hoofdvakken plantenfysiologie (bij dr.ir J.J.S. van Rensen) en landbouwplantenteelt (dr.ir. J. Vos). Met het behalen van het doctoraalexamen in november 1990 werd deze studie afgerond.

Na zijn studie werd er tussentijds door bemiddeling van Carforum een opdracht uitgevoerd bij het bedrijf Rhizopon en in september 1991 werd er begonnen met een éénjarig NOP project (Na Doctoraal Onderzoeksproject) op de toenmalige vakgroep Plantenfysiologisch Onderzoek onder leiding van dr.ir. J.J.S. van Rensen. Aansluitend is hij in september 1992 begonnen als assistent in opleiding (AIO) bij de vakgroep Plantenfysiologisch Onderzoek van de Landbouwuniversiteit te Wageningen. De resultaten van het promotieonderzoek staan in dit proefschrift beschreven.