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Photoinhibition of photosynthesis without net loss of photosystem II components in *Populus deltoides*

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Abstract. The photoinhibition of photosynthesis was investigated on intact attached leaves and isolated thylakoid membranes of *Populus deltoides.* Our studies demonstrate that in intact leaves photoinhibition takes place under high irradiance which is more pronounced at higher temperatures. No net loss of Dl and other proteins associated with photosystem II (PSII) were observed even after 64 % photoinhibition suggesting that the degradation of polypeptides associated with PSII is not the only key step responsible for photoinhibition as observed by other workers. Electron transport studies in isolated thylakoid membranes suggested water oxidation complex as one of the damaged site during high light exposure. The possible mechanisms of photoinhibition without net loss of D1 protein are discussed.

Keywords. Photoinhibition; D1 protein; photosystem II; *Populus deltoides.*

1. Introduction

Photoinhibition is a gradual decrease in photosynthetic efficiency of plants exposed to light intensities higher than those experienced during normal growth. During the last decade studies of photosynthesis and photoinhibition in higher plants have been strengthened by advances in our knowledge and the development of new techniques for in *vivo* and *in vitro* investigations. Following the biochemical studies on chlamydomonas (Kyle *et al* 1984), the synthesis and degradation of the Dl protein of the photosystem II (PSII) reaction centre during photoinhibition has attracted most attention (Barber and Andersson 1992; Aro *et al* 1993). The involvement of Dl protein in photoinhibition was based on the observations that this protein turns over very fast at high light intensities and that the *de novo* synthesis of proteins during recovery is essential. Degradation of Dl protein during photoinhibition has been suggested to be due either to a serine-protease associated with PSII (Shipton and Barber 1991; Salter *et al* 1992) or with a H_2O_2 induced hydroxyl radical attack (Sopory *et al* 1990) on a susceptible peptide bond. A limitation of these observations is that they are based on *in vitro* studies and do not provide any insight, into how the Dl is synthesized and integrated into the defective PSII assembly enabling recovery from photoinhibition.

In intact photosynthetic tissue, there is the complicating factor of repair occurring concurrently with the damage which is not possible in isolated system. It is known that the observed photoinhibition under high light conditions is the net result of damage

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and repair of the Dl component of PSII. Studies on intact leaves show that there is no net change in Dl protein levels even under photoinhibitory conditions (Cleland 1988; Aro *et al* 1990; Oquist *et al* 1992; Leitsch *et al* 1994; Schnettger *et al* 1992; Russell *et al* 1995) unless translation is inhibited by an inhibitor (Schuster *et al* 1988; Kettunen *et al* 1990). The assumption involving co-regulation of high rates of synthesis and degradation of the Dl protein *in vivo,* challenges the concept of Dl protein degradation as the only important step leading to photoinhibition. In addition to changes in the Dl levels observed in *in vitro* studies, reports on the damage of water oxidation complex (WOC) (Wang *et al* 1988; Chaturvedi *et al* 1992), PSI (Powles 1984; Satoh and Fork 1982; Inoue *et al* 1989; Terashima *et al* 1994) and involvement of P680 during photoinhibition (Cleland 1988) also exist.

Each of the group working on photoinhibition has developed their own interpretation based on the studies on two entirely different systems (*in vitro* and *in vivo*) which differ from each other to a great extent. We have studied photoinhibition in intact leaves as well as in isolated thylakoid membranes from a tree species, *Populus deltoides* which grows even in high light and high temperature and is a fast growing tree. Our data indicate that, during photoinhibition there is no net loss of components of PSII reaction centre under *in vivo* conditions as shown in other plants. The data further suggest distinct possibilities of damage of WOC and/or PSI component, resulting in photoinhibition.

2. Materials and methods

One year potted poplar (*Populus deltiodes* L., clone D121) plants were raised from cuttings and grown in 1000 cm^2 pots containing garden soil. The plants were watered daily and grown under natural irradiance and temperature.

2.1 *Light and temperature treatments and photosynthesis measurements*

Different light and temperature treatments to intact attached leaves of poplar plants were given in a laboratory built gas exchange system as described earlier (Trivedi *et al* 1992) with some modifications. The difference between the CO_2 concentration in the air entering in and exiting from the cuvette (ADC, UK) containing leaf was measured in ppm using LiCOR-6200 portable photosynthesis system. For all gas exchange measurements 3 to 4 leaves were used and calculations for the photosynthetic rates were made as described by van Caemmerer and Farquhar (1981). Before photoinhibitory treatments, photosynthetic rate of the leaf was measured at optimum conditions of PPFD (1000 μ mol/m^2/s) and temperature (30°C). Two leaves preselected for similar photosynthetic efficiency from the same plant were used for treatments. One of them was treated at optimal conditions for 3 h and the other leaf, in a separate cuvette, was subjected to different light and temperature treatments for the same period. After the treatment, photosynthetic rates of both the leaves were measured and percentage of photoinhibition was calculated.

2.2 *RNA isolation and northern analysis*

After treatment, total RNA from the leaves exposed to optimal and photoinhibitory conditions of light and temperature was isolated according to the procedure described

by McDonald *et al* (1987). For northern analysis, total RNA (20 μg) was electrophoresed under denaturating conditions, transferred to Zeta probe membrane (BioRad), prehybridized, hybridized with radiolabeled *psb*A and *psb*D gene probes, washed, exposed to X-ray films and developed according to the method described earlier (Sane *et al* 1994).

2.3 *Thylakoid membrane isolation and western analysis*

Thylakoid membranes from leaves treated at optimal and photoinhibitory conditions were isolated according to Kuwabara and Murata (1982). Total protein content was estimated according to modified procedure of Lowry (Peterson 1977). Protein (30 μg) was loaded on a 12·0% SDS-PAGE was carried out according to Laemmli (1970). The western blots were prepared by transferring the thylakoid membrane proteins from gel to PVDF membrane using mini-transblot apparatus (BioRad). Western blots were probed with antibodies specific to PSII components i.e., anti-D1, anti-CP43, anti-CP47 and anti-LHCP (a kind gift from Udo Johanningmeier, Germany). The colour development was performed by using goat-anti-rabbit alkaline-phosphatase conjugate (Bangalore Genei) with a mixture of nitroblue tetrazolium and dichlorophenolindophenol.

2.4 *Electron transport measurements*

Various photosynthetic electron transport reactions of thylakoids were measured in a temperature controlled Clark type chamber with adjustable volume (Hansatech, Britain). All the measurements were made in terms of either the uptake or evolution of oxygen. The artificial electron-donor-acceptor system was used for measurements of whole chain and partial electron transport reactions as described earlier (Sane *et al* 1984; Singh *et al* 1990) using 20 μg of chlorophyll/ml in the reaction mixture. Electron transport rates were corrected for temperature effect on solubility of O_2 in the buffer at different temperatures. The chlorophyll content of the thylakoid preparations was measured according to Arnon (1949).

3. Results

3.1 *Photoinhibition in intact leaves*

The response of intact, attached leaves of P. *deltoides* under different light and temperature treatments is given in table 1. The results show that poplar leaves could withstand the effect of high temperature at optimal light conditions i.e., 40°C and 1000 μmo1/m^2/s respectively and only a decrease of 16% was observed in photosynthetic rates. At high light (2000 μmol/m^2/s) and normal temperature (30°C), 38% of photoinhibition was observed. However, under high light and high temperature (2000 μmol/m^2/s and 40°C), 64% photoinhibition was observed. These results indicate that high light alone can cause photoinhibition but photoinhibition is more pronounced at higher temperatures. The inhibited rates, even when photoinhibition was 64%,

Table 1. Response of intact leaves under different light and temperature treatments.

Treatments			Photosynthetic rate ($\mu mol/m^2/s$)		
Light ($\mu mol/m^2/s$)	Temp. (°C)	Time (h)	Before treatment	After treatment	Photoinhibition (%)
1000	30	3	9·0	9·0	0
1000	40	3	9·0	7·6	16
2000	30	3	10·0	6·2	38
2000	40	3	9·0	3·4	64

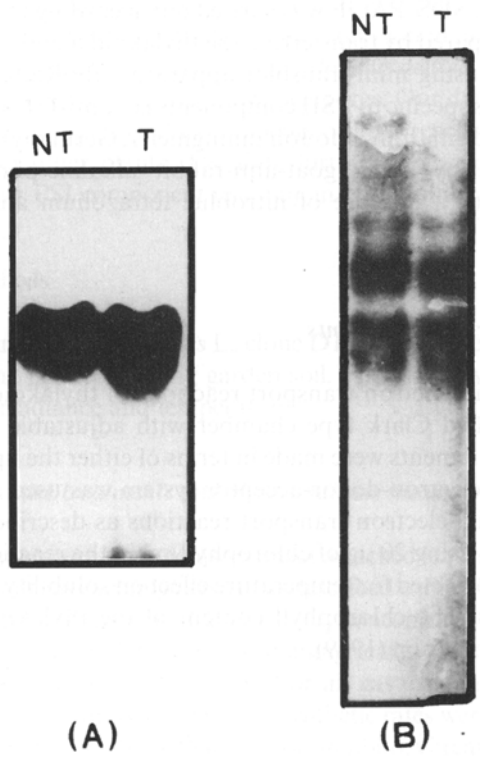

Figure1. Northern blot demonstrating steady state levels of *psb*A and *psb*D transcripts of *P. deltoides* during photoinhibition. Leaves of same photosynthetic capacity were exposed to optimum (NT)andphotoinhibitory(T) conditions i.e.,1000 µmol/m^2/s PPFD, 30°Cand2000 µmol/m^2/s PPFD, 40°C respectively. Total RNA (30 µg) isolated from these leaves was separated on 1·2% denaturating formaldehyde agarose gel, transferred to membrane and hybridized with radiolabelled psbA (A) and *psb*D (B) gene probes.

recovered to 85% when the leaves were transferred to low light (150µmol/m^2/s at 30°C) condition (data not shown). This suggests that photoinhibition could be induced in the leaves reversibly, such that they recovered from photoinhibition after removel of stress conditions.

3.2 *Photoinhibition and transcript levels of psbA and psbD*

Analysis of the transcripts of the *psb*A and *psb*D genes encoding Dl and D2 proteins of PSII was carried out in leaves treated with optimal and photoinhibitory conditions (figure 1). While *psb*A as expected showed only one transcript the *psb*D showed more transcripts of different sizes reflecting the processed and unprocessed transcripts arising out of *psb*DC operon. However, in both the cases the intensities of the observed transcripts were not qualitatively or quantitatively much different in photoinhibited and non-photoinhibited plants. It thus appears that the transcript levels of the Dl and D2 encoding genes remain more or less unaffected.

3.3 *Steady state levels of PSII polypeptides during photoinhibition*

Many groups have already shown that Dl protein is affected when isolated chloroplasts are illuminated at high light conditions (Aro *et al* 1993). To know, whether, such a possibility exists in intact leaves of poplar, we isolated thylakoid membranes from leaves treated with optimal and photoinhibitory conditions, electrophoresed, transferred to supporting media and immunoprobed with antibody specific to Dl protein (figure 2A). No qualitative or quantitative changes were observed in Dl protein levels in photoinhibited leaves in comparison to leaves treated with optimal conditions of light and temperature. These results suggest that in intact leaves under high light and high temperature stress conditions, a decrease in photosynthetic rate up to 64% is not

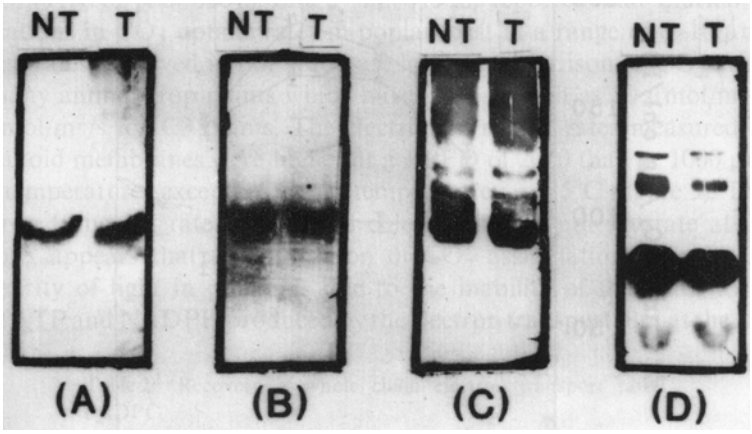

Figure 2 Immunoblot demonstrating steady state levels of PSII proteins from thylakoid membranes of *P. deltoides* during photoinhibition. Leaves of same photosynthetic capacity were exposed to optimum (NT) and photoinhibitory (T) conditions i.e., 1000 μmol/m^2/s PPFD, 30°C and 2000 μmol/m^2/s PPFD, 40°C respectively. Thylakoid membrane (30 μg) isolated from these leaves was separated on 12% SDS- PAGE and polypeptides were identified by immunoblotting with anti -Dl **(A),** anti-CP43 **(B),** CP47 **(C)** and LHCP **(D)** antibodies.

associated with any qualitative or quantitative change in D1. Analysis of other proteins associated with PSII, namely CP47, CP43 (chlorophyll *a* binding proteins) and LHCP was carried out by western blot analysis using antibodies specific to these proteins (figures 2B, C, D). No qualitative and/or quantitative changes were observed in CP47 and CP43 proteins under highlight and high temperature conditions. In case of LHCP, the photoinhibited samples did show somewhat reduced levels of this protein but it was not as much as the decrease in the rate of photosynthesis (64%). In case of CP47 and LHCP we observed one major band but other bands arising out of the nonspecific recognition by the antibodies also were apparent. These additional bands are not due to the degradation of the protein because they are at positions indicating higher molecular weight proteins. The antibodies used were polyclonal and may recognize some other polypeptides or undissociated polypeptides. Such observations have been made earlier (Hofer *et al* 1992) and the presence of these bands was suggested to be due to protein aggregation.

3.4 *Role of electron transport components in photoinhibition*

The above described results clearly demonstrated that the levels of PSII proteins remained unchanged during photoinhibition. To understand if the electron transport rates had decreased and if so which component was restricting the electron transport, whole chain and partial electron transport rates were studied using uncoupled thylakoid membranes. The optimal rates of photosynthetic electron transport of whole chain reaction ($H_2O \rightarrow MV$) at 1000 and 2000 μmol/m^2/s PPFD was observed at 30° C i.e., 150 nmol O_2/μg chl/h and 180 nmol O_2/μg chl/h respectively. In case of P P F D of

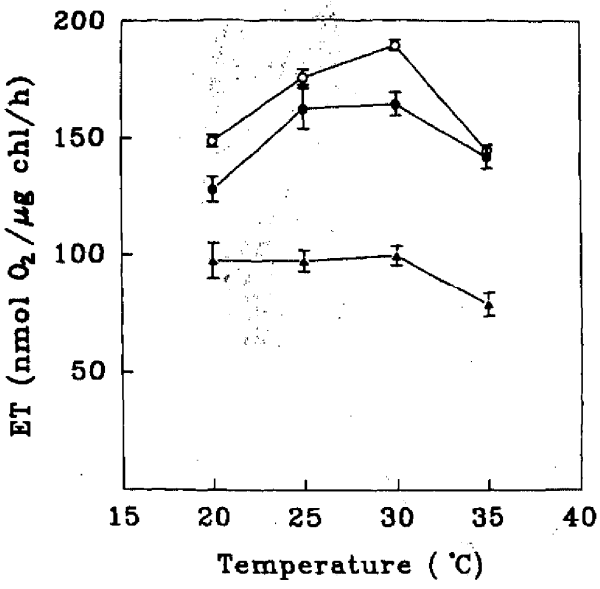

Figure 3. Rates of uncoupled whole chain electron transport ($H_2O \rightarrow MV$) of *P. deltoides* thylakoid membranes at different temperatures. Electron transport rates were monitored at PPFD 1000 μmol/m^2/s(●) and 2000 μmol/m^2/s (○) using 20 μg of chl/ml of reactionmixture. In another case, thylakoid membranes were first irradiated at PPFD 2000 μmol/m^2/s for 3 min and rates monitored under the same conditions (▲).

1000 μmol/m^2/s, not much loss in activity was observed when the temperature was raised from 30 to 35°C whereas considerable loss in activity was observed at PPFD of 2000 μmol/m^2/s with increase in temperature. At the same time when the thylakoids were treated initially at 2000 μmol/m^2/s of PPFD for 3 min, a loss in activity was observed at all the temperatures (figure 3) although the effect was more pronounced at higher temperatures. These results clearly demonstrated that at high temperature and high light, the electron transport activity was affected. It was thus clear that whole chain electron transport was decreased due to photoinhibition but steady state levels of polypeptides associated with PSII did not change at all. To evaluate whether any other components of electron transport instead of PSII were affected, a study of partial reactions was carried out. The electron transport to methyl viologen (MV) from diphenyl carbazide (DPC) which donates electrons to Z after water oxidation complex did restore electron transport rates but at higher temperature (35°C) the restoration was not complete (table 2). The partial restoration by DPC at higher temperature suggests that electron donation from the WOC to P680 had decreased during photoinhibition. It, therefore, appears that WOC is one of the sites damaged during photoinhibition. Since the restoration by DPC is not 100%, apparently some other sites are also damaged.

4. Discussion

In our observations maximum photosynthetic rates i.e., 9 μmol/m^2/s were obtained at a temperature of 30°C and PPFD of 1000 μmol/m^2/s. The optimum rates of CO_2 uptake in poplar compare favourably with rates of other species such as Rubus and Acer (11–15 μmol/m^2/s and 8–12 μmol/m^2/s respectively) and Acacia (8–12 μmol/m^2/s) (Trivedi *et al* 1992). Singh *et al* (1996) reported that diurnal and seasonal variations in CO_2 uptake rates in poplar exist in a range of 6–10 μmol/m^2/s. The highest rates observed in our study are low in comparison to CO_2 assimilation rates in many annual crop plants which have rates as high as 50 μmol/m^2/s for C4 and 30 μmol/m^2/s for C3 plants. The electron transport rates measured with isolated thylakoid membranes were higher at a PPFD of. 2000 than at 1000 μmol/m^2/s at all the temperatures except at higher temperature i.e., 35°C (figure 3). This shows that electron transport rates in isolated chloroplasts do not saturate at low light. It, therefore, appears that the saturation of CO_2 assimilation in intact leaves at lower intensity of light in poplar is due to the inability of the plant to use total amount of ATP and NADPH produced by the electron transport. But at the same time,

Table 2. Recovery of whole chain electron transport rates by DPC.

Temp. (°C)	Initial rate (nmol O_2/μg Chl/h)	Rates after 3 min treatments (nmol O_2/μg Chl/h)	
		− DPC	+ DPC
20	200	180	260
25	260	200	245
35	270	180	230

at high temperatures there is sharp decrease in electron transport rates at high light intensity suggesting some damage to the electron transport chain under those conditions.

The results indicate in *P. deltoides,* photoinhibition in intact leaves takes place under high irradiance which is more pronounced at higher temperatures. This observation is similar to those of Greer *et al* (1986) who also observed increase photoinhibiion at elevated temperatures. However, unlike in some previous reports the net levels of polypeptides associated with PSII remained unaffected in our studies. It is, however, not clear if the PSII is functional with full efficiency. Two types of PSII centres (Anderson and Melis 1983), PSIIα and PSII*β*, in appressed and non appressed thylakoid membranes respectively are present. It is possible that of these two centres the polypeptides associated with the fuctional PSII viz., PSIIα do undergo a change but this is not reflected in the net amounts as the newly synthesized polypeptides integrated in PSII*β* may compensate for this decrease. Although one may assume that there is no net change in the amounts of Dl in either of the PSII, we are not in a position to distinguish between these two possibilities. Neale and Melis (1991) have shown depletion of PSII*β* from non-appressed thylakoids of chlamydomonas during strong-irradiance.

The observation that levels of D1 have not changed during photoinhibtion is not surprising. Sundby *et al* (1993) have shown that high rate of Dl degradation occurs not only after photoinhibition but also under conditions where no net decrease in the functional PSII centres occurs. Aro *et al* (1990) have also reported that in isolated thylakoid membranes at low temperatures there was no degradation of Dl protein despite a pronounced decrease in electron transport. The repair cycle of PSII involves several steps including post translational processing, modification of proteins and association of the newly synthesized polypeptides with other electron transfer components in the thylakoid membranes which includes lateral movement of the Dl protein within the thylakoid.

Earlier reports have shown that the rate of recovery from photoinhibition is often too fast to be fully accounted for by protein synthesis and other steps yielding functional PSII (Somersalo and Krause 1989). Substantial and rather fast, recovery was seen even at chilling temperatures, when Dl protein turnover can be assumed to be negligible suggesting that the synthesis of new Dl is not crucial for the recovery (Somersalo and Krause 1990). Leitsch *et al* (1994) have studied the kinetics of recovery and suggested that it has two distinct phases. The first phase namely the fast recovery phase usually was completed within 20–60 min without Dl turnover. The subsequent slow phase proceeded for several hours leading to almost complete reactivation of PSII. Preincubation of leaves with inhibitor of chloroplast-encoded protein synthesis, inhibited the slow recovery phase only, indicating the dependence of this phase on resynthesis of the reaction centre protein Dl (Leitsch *et al* 1994). In their studies they also demonstrated that when streptomycin was absent, no degradation of the Dl protein could be detected upon photoinhibitory irradiance.

In recent studies phosphorylation/dephosphorylation of Dl has also been shown to play an important role during photoinhibition. Accumulation of phosphorylated form of the Dl protein, which degrades slowly than non-phosphorylated Dl during exposure to strong light under *in vivo* conditions is postulated to be involved in the regulation of Dl protein degradation and therefore the whole PSII repair cycle. Rintamaki *et al* (1995) have screened for the presence of phosphorylated form of Dl in thylakoid membranes from widely different plant groups. In their studies, in addition to

pumpkin (dicotyledon), phosphorylated form of D1 appeared during illumination in the thylakoids of wheat (monocotyledon) and pine (gymnosperm). On the other hand, no phosphorylated D1 was induced in a moss, a fern or a liverwort. This suggests that the differences in susceptibility to photoinhibition between higher and lower plants and phosphorylation of D1 protein may have evolved together. The fast recovery process suggested by Leitsch *et al* (1994) could be associated with the dephosphorylation of D1 protein which is the active form. Thus there is no need of resynthesis of new D1 for the fast recovery as the resynthesis takes longer time to assemble the functional PSII. It has been reported (Giardi *et al* 1994) that phosphorylation of D1 slows down the rate of electron transport whereas phosphorylation of CP43 and 10 kDa polypeptide provides protection against photoinhibition. It thus appears that phosphorylation of D1 may play an important role in photoinhibition and warrants further investigations.

Our studies for the other proteins associated with PSII suggest no substantial loss or degradation of CP47, CP43 and LHCP polypeptides. These results are in contrast to earlier report by Mori and Yamamoto (1992) who demonstrated the loss of D 1, CP43, CP47 and 10 kDa phosphoprotein during donor side photoinhibition. In their recent studies (Mori *et al* 1995) they suggested that although the loss of CP43 was most prominent among the above mentioned proteins, its degradation product was not detectable. The differences between our observations and by Mori *et al* (1992, 1995) may be due to the use of isolated thylakoids by them in which the synthesis of proteins to repair the electron transport complexes from photoinhibition is absent. Electron transport studies carried out by us suggest that there are other components of the electron transport chain e.g., WOC and PSI that may be damaged. Thus conclusions arising from *in vitro* studies which suggest that degradation of D1 is responsible for photoinhibition may not hold true for *in vivo* systems. Modifications/degradation of other components may also be involved in photoinhibition under *in vivo* conditions.

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